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Departamento de Biologia Animal

Characterization of the toxic potential of nanomaterials using *in vitro* cell models

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Dissertação

Mestrado em Biologia Humana e Ambiente

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Characterization of the toxic potential of nanomaterials using *in vitro* cell models

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Resumo

Os nanomateriais são estruturas com uma ou mais dimensões inferiores a 100 nanómetros. Devido à sua pequena dimensão, as nanopartículas apresentam atributos únicos, tais como a sua elevada área superficial relativamente à sua massa, reactividade ou força tênsil. Estas características influenciam grandemente algumas das propriedades dos nanomateriais, como a sua hidrofobicidade, carga ou toxicidade.

As propriedades das nanopartículas tornam-nas também muito úteis para o Homem, sendo aplicadas em medicina, farmácia, electrónica, cosmética, vestuário e biotecnologia, entre outras. O aumento de produção e utilização de nanomateriais tem vindo a aumentar também a possibilidade de exposição humana a este tipo de partículas, levando a preocupações relativas ao risco de toxicidade aguda ou crónica. A exposição humana pode ocorrer por diversas vias, sendo as mais relevantes a via inalatória, ingestão ou contacto com a pele. Dependendo do material e do órgão-alvo, a exposição a nanomateriais pode conduzir a diferentes consequências biológicas: a nível dos órgãos, os nanomateriais podem levar a inflamação ou a supressão do sistema imunitário e, a nível celular e molecular, a perturbações na estrutura e integridade do genoma, assim como a interacções com moléculas biológicas e inibição da actividade proteica, entre outras consequências.

Um dos nanomateriais mais utilizados são os nanotubos de carbono. Estes são constituídos por grafite cilíndrica disposta numa única camada (designados nanotubos de carbono de parede simples) ou em várias (nanotubos de carbono de parede múltipla). Os nanotubos de carbono apresentam propriedades como resistência e condutividade que os tornam muito úteis em aplicações como aparelhos electrónicos, vestuário ou biomedicina; cada vez mais, portanto, se torna provável a exposição ocupacional ou ambiental a este material. A semelhança estrutural destas partículas com fibras de amianto conduziu a questões relativas à sua segurança, pelo que já foram elaborados diversos estudos relativos aos seus efeitos biológicos. Alguns trabalhos sugerem que os nanotubos de carbono têm a capacidade de produzir toxicidade associada a lesões físicas, à produção de danos oxidativos por interacção com mecanismos celulares, ou a morte celular. Outros trabalhos defendem que estas partículas não causam toxicidade relevante.

O projecto de dimensão europeia “NANoREG” surgiu da necessidade de ser desenvolvida legislação e regulamentação apoiadas em conhecimento científico e adequadas à produção e ao uso actual de nanomateriais.

Este trabalho teve como objectivos principais a determinação do potencial cito- e genotóxico de um conjunto de nanotubos de carbono de parede múltipla (designados NM-400 a NM-403), e a consequente tentativa de associar este potencial às características físico-químicas dos nanomateriais. Com este

objectivo, a exposição por via inalatória foi analisada, pelo uso de duas linhas celulares *in vitro* provenientes de tecidos do tracto respiratório: epitélio pulmonar (células A549) e epitélio brônquico (células BEAS-2B).

A citotoxicidade dos nanotubos de carbono foi analisada com base em três parâmetros. Em primeiro lugar, as células foram contadas após a exposição aos nanomateriais utilizando o corante azul de tripanao para excluir as células inviáveis; a contagem foi realizada 3 e 24 horas após a exposição das células aos nanotubos. Os resultados deste ensaio apontam para a ausência de citotoxicidade após a exposição mais curta, e dados inconsistentes após a mais longa. Em segundo lugar, foi realizado o ensaio clonogénico, que se baseia na capacidade das células de se dividirem após a exposição ao agente em estudo. Este ensaio só foi realizado nas células A549 pois as BEAS-2B não permitem a formação de colónias. Os resultados apontam para uma citotoxicidade após a exposição a todos os nanomateriais, cuja intensidade se relaciona directamente com o tamanho das partículas, assim como ao seu diâmetro e área de superfície. Em terceiro lugar, foram calculados dois índices de viabilidade no ensaio dos Micronúcleos, cujo objectivo é avaliar se as células se dividiram durante a exposição aos nanomateriais em comparação com o controlo, e cujos resultados apresentam incoerências em relação aos outros já referidos. Estes dados podem ser justificados pelas diferenças existentes entre os ensaios, como o tempo de exposição ou a densidade celular.

Os efeitos genotóxicos dos nanomateriais foram avaliados com recurso aos ensaios do cometa e dos micronúcleos. O primeiro detecta lesões pequenas e reversíveis nas cadeias de DNA, ao passo que o segundo detecta efeitos irreversíveis ao nível cromossómico, tais como quebras ou perdas de cromossomas. Os resultados do ensaio do cometa sugerem que nenhum dos nanomateriais testados é genotóxico, uma vez que em ambas as linhas celulares e em ambos os tempos de exposição, os resultados são negativos. O ensaio dos micronúcleos, por outro lado, aponta para existência de genotoxicidade de dois dos nanomateriais (NM-401 e NM-402) nas células A549, mas não em células BEAS-2B.

Uma possível explicação para estes dados aparentemente contraditórios pode residir na hipótese de estes nanotubos de carbono serem compostos com efeitos aneugénicos, mas não clastogénicos: o ensaio dos micronúcleos permite a detecção de ambos os mecanismos de acção, ao passo que o ensaio do cometa só revela a quebra de cadeias de DNA. Outra justificação para os resultados é a possível influência da perda de viabilidade das células analisadas. Com base nos dados do ensaio clonogénico, estas partículas apresentam elevada citotoxicidade, pelo que os resultados dos ensaios de genotoxicidade, em particular do Ensaio do Cometa, poderão ser afectados por estes efeitos.

O meio de cultura usado para expor as células aos nanomateriais também é um parâmetro muito relevante na sua toxicidade. Neste trabalho, foram usados meios de cultura com proteínas, que podem ser adsorvidas pelas partículas e formar uma “corona” em seu redor; este processo pode alterar propriedades importantes dos nanomateriais, entre os quais o seu potencial efeito biológico. Também o método usado para conseguir uma dispersão homogénea de nanomateriais pode conduzir a diferenças nos resultados dos

ensaios de toxicidade. Neste estudo, foram observados alguns problemas relativos à perda de homogeneidade das dispersões de nanotubos de carbono, o que pode ter conduzido a que as células fossem expostas a massas de partículas de grandes dimensões conjuntamente com partículas individualizadas. O período durante o qual as células são expostas ao nanomaterial é também um aspecto essencial na produção de efeitos tóxicos.

Resumindo, este projecto forneceu informações relativas à toxicidade dos nanotubos de carbono que, complementadas pelas conclusões dos restantes parceiros do projecto europeu, poderão contribuir significativamente para a avaliação de risco e criação de legislação relativamente à utilização de nanomateriais. Na linha celular BEAS-2B, nenhum destes nanomateriais parece produzir efeitos tóxicos, quer a nível de célula, quer a nível de genoma, nas condições experimentais utilizadas. Nas células A549, por outro lado, os três nanomateriais testados parecem ser acentuadamente citotóxicos, e dois deles (NM-401 e NM-402) são também genotóxicos.

Em relação a perspectivas futuras, pode-se concluir que nem todos os ensaios de toxicidade existentes actualmente são adequados à análise de nanopartículas, pelo que novas metodologias devem ser desenvolvidas e complementadas por ensaios *in vivo*. Todos os estudos envolvendo nanomateriais deverão também descrever as características físico-químicas dos materiais usados, de forma a se poderem comparar os resultados com os de outros trabalhos.

Palavras-chave: nanomateriais, nanotubos de carbono, genotoxicidade, citotoxicidade.

Abstract

Carbon nanotubes are strong and flexible fibers that have a broad range of applications, such as in electronic devices, mechanical industry and medical procedures, among others. However, the use of these materials can also have consequences to human life and to the environment, as they may cause tissue inflammation, asthma or cancer. The general objective of this project was to provide some scientific evidence about the risks related to the use of carbon nanotubes, so that relevant legislation could be elaborated. More specifically, the study aimed at characterizing the cyto and genotoxicity of several carbon nanotubes. Cell counting coupled to the Trypan Blue Exclusion and the Clonogenic assays were performed to assess the cytotoxicity of a series of multi-walled carbon nanotubes (NM-400 to NM-403) in human lung (A549) and bronchial (BEAS-2B) epithelial cell lines, and the Comet and the Micronucleus assays were employed to characterize their genotoxicity.

The results pointed to a substantial difference in the nanomaterials toxicity in the two cell lines. In the BEAS-2B cells, no cytotoxicity or genotoxicity was produced by any of the nanotubes. In A549 cells, on the other hand, significant cytotoxicity was produced by three of the carbon nanotubes (NM-401 to NM-403); the dose-response pattern appeared to be associated to the particle's length, diameter and surface area. It is suggested that this may be related to physical aggression or to damage to the membranes of the cells by the particles. In terms of genotoxicity, the two longer nanotubes (NM-401 and NM-402) caused significant concentration-related chromosomal damage; a direct correlation between the aspect ratio of the particles was significant association to its genotoxicity. This may be related to interference of the particles in common biological processes, or to the generation of oxidative stress.

In the future, a standardized protocol should be used and a comprehensive list of characteristics should be provided in every study regarding nanomaterials, since parameters such as dispersion quality, medium protein content and properties of the particle are very influential in the toxicity of nanomaterials.

Keywords: Nanomaterials, carbon nanotubes, genotoxicity, cytotoxicity.

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A549 – human epithelial lung adenocarcinoma cell line

ATCC – American Type Culture Collection

BEAS-2B - human epithelial bronchial cell line

BEBM – Bronchial epithelial basal medium

BEGM – Bronchial epithelial growth medium

BSA – Bovine serum albumin

CBPI – Cytokinesis-blocked proliferation index

CNT – Carbon nanotube(s)

DAPI - 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

EDTA - Ethylenediamine Tetraacetic Acid

EMS – Ethyl Methanesulfonate

FBS – Fetal bovine serum

FBSi – Heat-inactivated fetal bovine serum

FP7 – Framework program 7

FPG – Formamidopyrimidine DNA Glycosylase

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid

HPLC – High-Performance Liquid Chromatography

IC50 – Half maximal inhibitory concentration

INSA – Instituto Nacional de Saúde Doutor Ricardo Jorge (National Institute of Health, Portugal)

LDH – Lactate Dehydrogenase

MMC – Mitomycin C

MNBNC – Micronucleated binucleated cell(s)

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MWCNT – Multi-walled carbon nanotube(s)

NM-400 to NM-403 – nanomaterial 400 to 403, as designated by Joint Research Centre

PBS – Phosphate Buffered Saline

RI – Replication index

ROS – Reactive oxygen species

RPMI – Roswell Park Memorial Institute

SD – Standard deviation

SEM – Scanning electron microscopy

SSC – saline-sodium citrate buffer

SWCNT – Single-walled carbon nanotube(s)

TEM – Transmission electron microscopy

Introduction

1. Nanomaterials' properties and biological interactions

Nanomaterials are natural or manufactured structures with unique properties, the most relevant being their size: at least one of their dimensions is inferior to 100 nanometers. Other characteristics – such as shape, surface area, chemical and biological reactivity, magnetism, conductivity, strength, resistance or charge – differ greatly from those of the same material in other dimensions [1-10].

These characteristics, however, have a great influence over some of the properties of the nanoparticles. Since most of the nanomaterials are hydrophobic and/or charged, they are easily attracted to one another and have a tendency to form aggregates or agglomerates (particles are held together by strong chemical bonds of by weak van der Walls forces, respectively [11]). This dynamic behavior alters the size of the particles and, consequently, may cause a variation of the other properties (particularly surface area and biological reactivity) [12-14].

Another important aspect to consider is that impurities that adhere to the exterior of the particle (resulting from air or water contaminants or residual materials left behind during the synthetic process) can change its properties, altering the charge or reactivity of the nanomaterial [6, 13].

In addition, when a biological system is exposed to nanomaterials, the particles have to travel through complex pathways inside the organism, subjecting them to very diverse environments, with specific biochemical conditions (protein composition, pH, enzymatic activity, metal composition, etc.) [12]. The small size and large aspect ratio of the particles cause them to easily interact with biological molecules in those environments, such as proteins, nucleic acid or lipids – depending on the type of particle and biomolecule, their characteristics, the agglomeration/aggregation state, the pH of the medium, etc. Consequently, some nanoparticles (such as metal oxides and carbon nanotubes) have the capacity to adsorb proteins, creating a "protein corona" – which may happen instantly when the nanoparticles contact with the biological medium. This corona may alter some of the particles' properties, such as size, reactivity, charge or ability to aggregate/agglomerate or to bind to other proteins, as well as the preferred route inside the cells and the organism; besides, throughout the life cycle of the particle, the corona proteins may detach and contaminate the surrounding environment, possibly causing negative effects (which may, erroneously, be attributed to the nanoparticles) [1, 6, 12, 13]. Conversely, the formation of a protein corona around the nanoparticles may affect the proteins' properties, such as secondary structure, flexibility, chemical composition and thermodynamic stability; these characteristics also influence the strength of the nanoparticle-protein association and the rate of binding and unbinding [12]. The adsorption of proteins to the nanoparticles may affect other events downstream, such as interactions between proteins, cellular signaling, DNA transcription and loss of enzyme activity – ultimately, it may lead to cellular

damage, apoptosis or abnormal immune response [12]. Gold nanoparticles and zinc oxide, for example, have been reported to cause conformational changes in bovine serum albumin, which does not happen with carbon C60 fullerenes; titanium dioxide reduces polymerization of tubulin, influencing cellular structure [12].

However, since the protein corona may, in fact, enhance the uptake of nanoparticles into the cells, and some nanoparticles have the ability to cross largely impermeable membranes such as the blood-brain barrier, nanotechnology represents a new possibility in the field of biomedical applications: the efficiency of the transport into the brain may be drastically increased by binding drugs and specifically targeted proteins to these nanoparticles, possibly enabling the treatment of neurological diseases [12, 15].

All of these properties have made nanoparticles very useful in several fields of human life [2, 3, 6], and the number of uses for nanotechnology keeps increasing. The most prominent field is medicine – nanoparticles are used in bioimaging, drug delivery, cancer therapy, fluorescent labels, and diagnostic agents, among others [16] (described in greater detail in Table 1).

Table 1. Biomedical applications of some nanomaterials [15].

Nanomaterial		Uses
Metallic nanoparticles	Iron oxide nanoparticles; manganese oxide nanoparticles; crystals of gadolinium oxide; metal nanoshells; quantum dots	Tumor targeting, analysis and therapy. Contrast agents in optical imaging. Treatment for gliomas. Quantum dots can also be used as a dye in fluorescence-based bioanalytical techniques
Carbon Nanotubes	May have a large variety of applications depending on size.	Molecular therapy or immunotherapy: direct delivery of antigens to antigen-presenting cells or microglia in the central nervous system
Inorganic Nanoparticles	Ceramic nanoparticles; organosilicates; transition metal oxides; metalloids; metal sulfides	Drug carriers; protect the drug molecules and keep them from being denatured or degraded by the organism
Dendrimers	Highly branched macromolecules, with exterior end groups that can be functionalized by the attachment of specific molecules	Drug carriers, by encapsulation of drugs. Large variety of groups can be attached to the exterior of the nanoparticles, allowing for different properties and, consequently, different applications

Nanomaterials are also broadly used in other industries: pharmaceutical, automotive, aircraft, electronics, optics, ceramic, glass, paints, cosmetics, clothing, biochemical and environmental engineering, biotechnology, food, construction, etc. [10, 14, 17, 18] A short list of some of the main uses of nanomaterials in consumer products can be found in Table 2.

Table 2. General uses of some of the most common nanomaterials.

Nanomaterial	Uses	Reference
Metallic nanoparticles TiO_2, Cr_2O_3, Mn_2O_3, Fe_2O_3, NiO, CuO, ZnO, ZrO_2	Biochemical catalysts, electronic devices, water purification, cancer diagnostics and therapy, drug delivery, food additives, artificial dyes, cosmetics, medical and dental implants, sunscreens, biosensors, bioimaging (as contrast agents).	[19-28]
Silver nanoparticles	Textiles, cosmetics, health care, antibiotic agent in bandages and medical material.	[29-31]
Carbon Nanotubes	Drug carriers, rubber tires, pigments, electronics, catalysts, biosensors, photonics, tissue engineering, batteries, composites.	[30, 32-34]

The number of individuals working in all of these industries who are, therefore, potentially exposed to the nanomaterials is very high and constantly increasing, which is raising many questions concerning the safety of the nanomaterials. Also, a large number of industries using nanotechnology means a large number of products with nanoparticles imbedded in them, which may increase the environmental exposure of the general population too [5, 8, 35, 36].

During the nanomaterial lifecycle, from the synthesis to the disposal of the nanomaterials, there are many occasions in which environment contamination is also possible and, in some cases, even likely. Accidentally or deliberately, nanoparticles may reach the soil or courses of water, where they can deposit or react, possibly altering their properties, or be transported far from where they were originally produced or discharged. Agglomerates/aggregates will likely be formed, as well as bonds between nano- and metallic particles naturally present in the environment [14, 17].

There are several routes of human or animal exposure to the nanoparticles, such as inhalation of airborne particles (more likely and frequent in both occupational and environmental exposure), absorption of nanoparticles through the skin, ingestion of the materials included in food products, inoculation with pure or processed nanoparticles (relevant in medical applications) and through physical contact of the nanomaterials with cuts in the skin [1, 3, 7, 37]. It is, therefore, necessary to study the effect the different nanoparticles have in the human organism through each of these routes, since each of them may affect different organs or vital processes. However, it is possible that the nanoparticles not only have an effect on the more exposed organ (such as skin, lung or stomach), but also on the others: nanomaterials may be transported to different locations throughout the body, travelling through the blood circulation or the lymph system [1, 37]. They may then deposit in sensitive organs, such as bone marrow, lymph nodes, spleen, heart and central nervous system [1, 7, 10]. The distribution of the nanoparticles inside the organism, as well as the interactions with the cells depend greatly on the characteristics of the particles (size, shape, surface area, coating, agglomeration/aggregation, etc.) and the cell types in question [1, 4, 6, 32, 36].

The size of the nanoparticles is the most influential feature in terms of biological reactivity and toxicity, due to the increase in the ratio between surface area and mass of the particle. Their size also allows the particles to disperse throughout the entire organism and to penetrate several barriers and membranes, reacting with biomolecules and interfering with biological processes. Additionally, the increase in surface area leads to an increase in the release of free radicals and metal ions from the nanoparticle, which may interact with the cells and molecules. Other characteristics of the surface of the nanoparticles, such as charge or coating, may also influence greatly the biological properties of the material, particularly those relating to toxicity. Due to those changes to the particles, the binding of other molecules (or nanoparticles) may be enabled of inhibited, altering the way these particles are internalized, processed or eliminated from the organism. Agglomerated particles, for example, may lose the ability to enter the cells or the organelles due to the increase in size, reacting differently to biological systems than the non-agglomerated particles. It is not possible to extrapolate toxicity data from larger particles with the same chemical composition, since nanomaterials may have different properties, causing alterations in the uptake, distribution, metabolism and elimination of the particles [35].

Once inside the organism, the nanoparticles able to penetrate cell membranes mostly do it through endocytosis (phagocytosis or pinocytosis)[1] or electrostatic attraction, and may stay free in the cytosol or within phagosomes [12] – unlike most xenobiotics, which are degraded in lysosomes and exocytosed. Consequently, nanoparticles may react to cell organelles or enter the nucleus, interfering in the normal cell processes and damaging essential molecules, such as DNA and proteins [34]. Some particles may even be able to cross the nuclear membrane and enter the nucleus by diffusion across the membrane or by transport through nuclear pore complexes [35]. Besides, some nanoparticles are made from non-biodegradable materials, which give them the capacity to stay in biologic tissues for years after the original exposure [4, 6, 35, 36].

At the organ and tissue level, it is thought that nanoparticle exposure may result in inflammation, oxidative stress [6, 35, 38, 39], stroke, myocardial infarction, alterations in the permeability of the blood-brain barrier [37] and suppression of the immune system response.

2. Characterization of the Genotoxic Effects of the Nanomaterials

A genotoxic event is one in which a chemical molecule triggers a reaction which will, after a period of time, lead to a permanent change in the genome of the cell. Examples of genotoxic events are direct damage to the DNA molecule, interference with processes such as mitosis and DNA replication or repair and disruption of the normal function of enzymes and proteins. Depending on the type of damage and its extent, these events may be deadly to the cells, or may be repaired quickly (through base or nucleotide

excision repair or mismatch repair, for example). To the organism, these occurrences may result in reproductive defects, developmental abnormalities, genetic diseases and carcinogenesis [40, 41].

At the cellular level, nanoparticles may cause genotoxicity and, consequently, possibly lead to carcinogenesis. This genotoxicity may be caused by the direct interaction of the nanoparticles with the DNA molecules, or by damage from by-products of the nanoparticle biotransformation (such as reactive oxygen species or ions released from the particles). The first, called direct genotoxicity, disrupts processes such as replication and transcription, and affects the structure of the DNA molecule; at the chromosome level, nanomaterials may cause breaks and losses (clastogenesis and aneugenesis, respectively), both mechanically and by chemically binding to the molecules [35]. The latter is known as indirect genotoxicity. The nanomaterials interact with proteins and the mitotic structures, disturbing the processes of replication, transcription, repair and cell division. Protein activity may be inhibited or altered, and the DNA molecules may be affected by by-products of nanoparticle processing – such as reactive oxygen species (ROS), transition metals (such as Fe^{2+}, Ag^{+}, Cu^{+}, Mn^{2+}, Cr^{5+} and Ni^{2+}) and antioxidants – or remains of cell components – such as mitochondria (which, when damaged, can also produce ROS), inflammatory cells, ions and transcription factors. These by-products, particularly ROS, may attack the DNA molecules, and cause purine- and pyrimidine-derived oxidized base lesions (producing 8-OxoGuanine, for example, which is the most frequent product of purine oxidation, and is highly mutagenic) and DNA strand breaks [13, 35, 39]. Besides genotoxicity, nanoparticles can cause damage in other biomolecules and cell processes, such as methylation and phosphorylation; modification of proteins may lead to the silencing of genes and to changes in gene expression, altering the production and metabolism of proteins and the normal survival of the cell [35].

An extensive review of several assays that can be used to test nanomaterials for their geno- and cytotoxicity, as well as other useful data, can be found in Singh *et al.* [42]. As a concise review of this information, and focusing solely on the genotoxicity testing, the following list summarizes the main assays used to analyze the genotoxic effects of nanomaterials: the alkaline comet assay (or single cell gel electrophoresis assay), which detects the single- or double-strand breaks in DNA and, with modifications, other endpoints, such as oxidative damage; the cytokinesis-blocked micronucleus assay, which identifies chromosome instabilities, such as loss or fragmentation of chromosomes, as well as apoptotic or necrotic events, or even cytotoxicity; Ames test, which uses *Salmonella* bacteria to determine the capacity of a certain chemical to cause reversion of mutations; chromosome aberrations test, where the cell cycle is arrested at metaphase and the chromosomes are observed for structural or numeric alterations; detection of DNA adducts, such as 8-hydroxydeoxyguanosine, through HPLC or mass spectrometry based techniques [42].

Besides from these tests, other endpoints that do not involve genotoxic events include analysis of protein expression, phosphorylation and activity, testing cell viability and proliferation (with MTT assay,

Neutral Red assay, trypan blue staining of unviable cells, or analysis of the proliferation and replication indexes provided by the micronucleus assay), measuring glutathione production, analyzing cytokine activity, analyzing histological aspects, etc. [42]

The properties of the particles are key factors in their distribution, transport and toxicity; the coating and impurities, for example, may influence the particles' tendency to bind to one molecule and not to another, creating the possibility that a toxicity result may be attributable to the coating, rather than to the nanoparticle itself [6, 13]. Another example is the protein corona: it can increase the particles' mobility and their capacity to penetrate organs otherwise inaccessible, as well as affect their biological activity [6, 13]; besides, the transformation and degradation of the proteins attached to the nanoparticles (as they travel throughout the organism and possibly deposit in one or more organs) may affect these parameters even further, causing changes in the toxicity of the nanomaterials over time [13].

It is not clear whether any of these (or other) properties is singularly responsible for the possible toxicity of the nanomaterials, or if it is due to the combination of several [6, 35]. The majority of the authors have obtained conflicting results concerning the toxicological impact of nanomaterials; this is most likely caused by differences in the physicochemical properties of the studied nanomaterials (such as type, size, composition, shape, stability, coating, surface area, electrical charge, etc.) [6, 10, 32, 35], as well as to other variables inherent to the assays used and the characteristics of the exposure (dispersion method, exposure time, concentration, culture medium, etc.) [6, 32, 35]. The solvents used for the preparation of the nanoparticles solutions or dispersions, for example, may differ in pH, temperature or dissolved molecules (such as proteins or metals), altering the results obtained in the assays. The dispersion protocol – procedure intended to break apart the nanoparticle agglomerates or aggregates in order to obtain as many single particles as possible – is a large influence on the outcome of the assays, since it alters the size of the particles. The type of assay also influences greatly the obtained results regarding the toxicity of the nanomaterials – *in vitro* methods can mainly detect the primary genotoxicity, and *in vivo* assays may be able to detect secondary genotoxicity too, due to the influence of the immune system of the tested animals. Concerning the *in vitro* assays, the toxicity of the nanoparticles depends on cell line, since different cell types have distinct susceptibility, metabolic activity, DNA repair capacity and particle internalization properties, among other unique characteristics. The assessment of nanoparticle genotoxicity should be performed using several assays and endpoints, due to the fact that there may be more than one mechanism leading to the DNA and cellular damage [35].

3. Multi-walled Carbon Nanotubes and Genotoxicity

Carbon nanotubes (CNT) are nanoparticles with a unique structure and very attractive physical, chemical and electrical properties, such as high tensile strength and conductivity. They may be separated in three groups, depending on the structure of the material: if they have only one flat graphite layer, they are known as graphene; if they have only one graphite layer, but it is cylindrical, they are Single-Walled Carbon Nanotubes (SWCNT); if, on the other hand, they have several layers of cylindrical graphite, they are Multi-Walled Carbon Nanotubes (MWCNT) [39, 43] (see Figure 1).

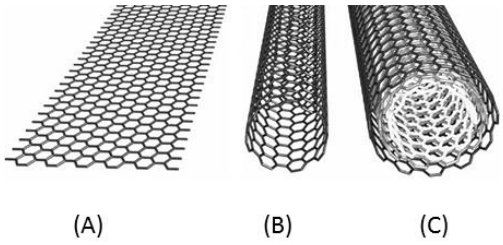


Figure 1. Graphic representation of carbon nanomaterials. (A) Graphene sheet; (B) Single-walled carbon nanotube; (C) Multi-walled carbon nanotube. [44]

CNT have a broad range of applications, such as in electronics, electrical appliances, batteries, clothing, biotechnology and composites [32, 34, 39, 43]; in biomedicine alone, CNT are used for diagnostics and therapeutics, as well as for recognition of antibodies, sequencing of nucleic acids, as biocatalysts of biological reactions, as components in regenerative surgery (in the central nervous system and in orthopedic interventions) and as drug delivery vectors [43].

However, CNT are hydrophobic and, therefore, difficult to solubilize in biological fluids; as a result, several different protocols have been proposed to disperse these particles [45-48]. In these procedures, CNT are often modified by binding them to chemical and/or biological molecules (such as proteins, polymers or surfactants), enhancing their solubility and bioavailability. These modifications are controversial, though, since they may alter properties of the nanoparticles other than their solubility, and may increase their toxicity [40, 43].

Due to CNT's small dimensions and low density, exposure through the respiratory route is likely, especially in occupational settings; if the particles have been modified as stated above, a reaction between the CNT and the biological molecules may happen, leading to a response. Besides, these particles are degraded slowly, and therefore can stay in the organism or in the environment for a long period of time after initial exposure [49].

These facts have raised some concerns regarding the possible toxicity of the CNT. Some authors have proposed a toxicity profile identical to that of asbestos, based on the similarity of shape between the two materials: the particles may penetrate the intrapleural space and cause inflammation and tissue fibrosis

[40, 49, 50]. Other authors suggest that the damage induced by the CNT to the DNA of the cells is related to direct mechanical injury (thus, associated to the physical characteristics of the nanotubes), and not to the production of ROS and oxidative damage [35]. There have been reports with discrepant results, some displaying genotoxicity and some not, possibly due to differences in the experimental methods used or in the characteristics of the particles (for example, contaminants or residues in the particles, such as metals) [32, 35, 40, 49].

Several *in vivo* studies reported that exposure to CNT cause granulomatous inflammation, pulmonary fibrosis, increased release of lactate dehydrogenase, cell hypertrophy and hyperplasticity, nuclear abnormalities in macrophages or disruption of the mitotic spindle, among other negative effects. Genotoxicity is a frequently observed consequence of CNT exposure, as well as increased proliferation of epithelial cells, which is a common feature of pulmonary carcinogenesis [40, 49].

In vitro studies have shown evidence of increased release of lactate dehydrogenase, and depletion of glutathione and superoxide dismutase – enzymes that protect the cells from oxidative damage. These effects indicate production of ROS; ROS may be formed due to the direct effect of the CNT inside the cell, or due to the internalization of the nanotubes by the mitochondria and subsequent mitochondrial dysfunction. This induces tissue inflammation, activation of cellular signaling pathways, DNA damage, chromosomal aberrations, abnormalities in the cell growth and ultimately, cell death; all these processes may lead to lung injury and carcinogenesis [39, 40, 49].

There are authors who propose yet another consequence of CNT exposure: nanotubes may be able to interact with microtubules [49]. Microtubules are dynamic polymers, formed by subunits of alpha and beta tubulin bound by non-covalent hydrogen bonds; when cell division occurs, the microtubules assemble at the centrosome and form the mitotic spindle, pulling the chromosomes to opposites sides of the cell. Throughout this process, the length of the spindle varies due to the activity of cellular motors (such as kinesin and dynein), which assures the correct segregation of the duplicated chromosomes into the two daughter cells. The CNT may disturb this process, inhibiting the activity of the cellular motors and disrupting the centrosomes; it may even occur the incorporation of nanotubes in the mitotic spindle (the two structures have approximately the same diameter; see Figure 2), preventing it from varying in size or shape, and not allowing the separation of the daughter cells. This theory offers an explanation for the number of abnormalities in number and size of chromosomes observed in cells exposed to CNT (micronuclei or multinucleated cells, for example) and, ultimately for the possible link to cancer [49].

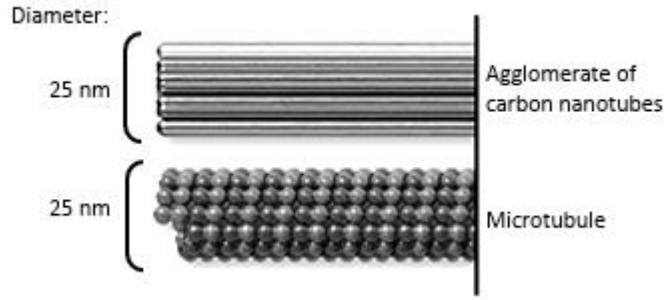


Figure 2. Comparison of the structures of an agglomerate of carbon nanotubes and of a microtubule. The straight lines on the agglomerate of CNT represent the individual fibers; the small spheres in the microtubule represent the alpha and beta tubulin. Adapted from [49].

4. The NANoREG Project

Considering the importance of nanotechnology in the current society and the effort made by laboratories world-wide to understand its toxic potential, it is essential to make the connection between the scientific body and the regulators and legislators, and to start making informed decisions concerning the safety of nanoparticles. With this purpose, the European Commission approved a project called *NANoREG - A common European approach to the regulatory testing of nanomaterials* (FP7/2007-2013, grant agreement 310584; *http://www.nanoreg.eu/*), which is inserted in the Seventh Framework Program (FP7) [51].

The main objective of NANoREG is to provide solutions for already existing problems concerning nanomaterials, providing regulators and scientists with the means to perform risk assessment, involving toxicity testing and exposure measurements. In the long term, the project aims to develop or adapt new protocols for testing the particles, as well as to establish a better collaboration between science, industry and regulators, in order to more efficiently manage the risk inherent to the utilization of nanotechnology [51].

A large number of European countries are contributing to NANoREG, such as Netherlands, Belgium, Germany, Denmark, France, Austria, the United Kingdom, Switzerland, Spain, Ireland, Sweden, Norway, Italy, Finland and Portugal. However, this project also intends to establish connections and associations with other countries, such as the USA, Canada, Australia, Japan and Russia, in order to globalize the standards to nanotoxicology testing and legislation [51].

In Portugal, the NANoREG project is represented by PToNANO, which is a consortium of four entities: the Institute for Welding and Quality, the National Institute of Health Doutor Ricardo Jorge, I.P (INSA), the Portuguese Institute for Quality, I.P and the Portuguese General Directorate of Health [51].

INSA, being the state health laboratory and having the mission of contributing to international knowledge and guidelines in public health, is involved in performing research concerning the toxicological

aspects of the nanoparticles, in the development of new analytical methods, and in the answering of questions related to the materials' safety. This data will be provided to the authorities and to the industry, in order to better develop legislation and solutions regarding occupational and environmental exposure to nanomaterials [51].

Objectives

The general objective of this project was to contribute to the assessment of the influence of the physicochemical properties (such as length, thickness and surface area) of a set of well characterized multi-walled carbon nanotubes on their cyto- and genotoxic potential, in order to understand the characteristic that is the most relevant to its toxicity.

The specific objectives of this project were: i) to test the genotoxic potential of three carbon nanotubes in human respiratory tract cells using *in vitro* methodologies (comet and micronucleus assays); ii) to test the cytotoxicity of these three nanomaterials (cell counting method and the clonogenic assay); iii) to try to disclose relationships between a certain physicochemical property and the toxicity of the carbon nanotubes.

Material and Methods

1. Cell Culture

There are many advantages in using *in vitro* cell models in toxicity testing of any chemical instead of *in vivo* systems, such as the reduction of the number of animals, monetary costs and time, as well as the existence of numerous well-characterized and available cell models. Besides, there is also the possibility to increase the complexity of the *in vitro* system, by including different cell lines in the study, in order to analyze different endpoints; by adding chemical agents (such as antioxidants, cell pathways inhibitors, or growth factors), it is also possible to alter several biochemical parameters and, therefore, study other mechanisms [52].

However, there are also disadvantages in the use of *in vitro* models, the most prominent being the limited number of different cell lines that can be combined in only one *in vitro* system; these systems, consequently, cannot adequately mimic the processes that happen *in vivo*, where several cell types interact, and the different tissues and organs may influence the cellular and molecular mechanisms. Another aspect to have in mind when using *in vitro* cell models is that the target organ for the exposure must be already known, since the toxicity testing will be focused on a single tissue; in addition, in *in vitro* systems, evidently, the influence of the immune system on the cellular and molecular mechanisms is inexistent [52].

The route of exposure and the potential target organ of a given particle or molecule are the main factors that define the *in vitro* cell types used in a toxicity study. However, this decision may be problematic, as different cell lines can produce different assay results, due to the fact that the molecular metabolism and toxicity response of cells from different tissues can change greatly. In addition, the characteristics of the cells when growing in culture may influence their susceptibility to the chemicals or particles, as their metabolism may be altered due to changes of medium or to cell density (the concentration of proteins or serum may cause morphological changes in the cells, and the achievement of confluence may inhibit the growth of some cell lines) [52].

The Bronchial Epithelium Cell Basal Medium (BEBM), as well as the growth supplements (BEGM SingleQuots) is from Lonza/Clonetics (Basel, Switzerland). The Hyclone Fetal Bovine Serum (FBS) is from Thermo Scientific (Waltham, MA, USA). All the other reagents used in cell culture – phosphate buffer saline (PBS 1X; without calcium, magnesium or phenol red), trypsin-EDTA 0.05% (1X), RPMI-1640 Medium (1X) + GlutaMAX (RPMI-1640), the heat-inactivated Fetal Bovine Serum (FBSi), Dulbecco's Modified Eagle Medium (DMEM; with 1 g/L glucose, L-glutamine and pyruvate), HEPES buffer solution (1M), amphotericin B (Fungizone; 250 µg/mL), Penicillin/Streptomycin mix (Pen/Strep; with 10000 units/mL of penicillin and 10000 µg/mL of streptomycin) and Trypan Blue Stain (0,4%) – are from Gibco (Scotland, UK).

Considering the aim of this project, the chosen cell lines were the BEAS-2B and the A549, both isolated from human respiratory tract tissues.

The BEAS-2B cell line was obtained from the American Type Culture Collection (ATCC No. CRL-9609; see Figure 3); it was isolated from healthy human bronchial epithelium, and was obtained from the autopsy of non-cancerous individuals. Later, the cells were infected with an adenovirus 12-SV40 virus hybrid (Ad12SV40), and then cloned. These cells display adherence properties, and are suitable hosts for transfection processes [53].

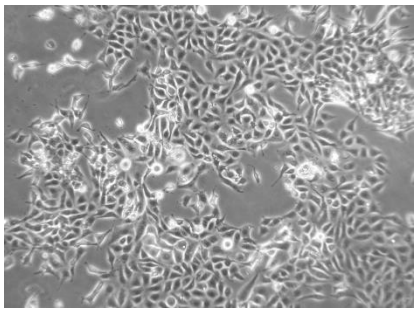


Figure 3. BEAS-2B cells in BEGM culture medium.

Due to their main purpose – being a protective barrier to the bronchial tissue – these cells produce and release important immunological molecules, such as lipid mediators, inflammatory enzymes and cytokines [54]. For this reason, and since this cell line retains the ability to differentiate when exposed to serum, it is often used to analyze chemical and biological agents, testing for potential to induce or affect events of differentiation and/or carcinogenesis in pulmonary tissue [53].

The growth medium used for the BEAS-2B cell cultures was the serum-free Bronchial Epithelium Growth Medium (BEGM), which consisted of 500 mL of BEBM and 6 mL of BEGM SingleQuots: 2 mL of bovine pituitary extract, 0.5 mL of hydrocortisone, 0.5 mL of human epidermal growth factor, 0.5 mL of epinephrine, 0.5 mL of transferrin, 0.5 mL of insulin, 0.5 mL of retinoic acid, 0.5 mL of triiodothyronine and 0.5 mL of gentamycin/amphotericin B. The cells were maintained in culture flasks in an incubator at 37 ºC, in 5% CO_2.

When the cells reached a confluence of 80%, a subculture was performed: the cells were washed with preheated sterile PBS and incubated for 5-7 minutes with trypsin-EDTA in an incubator at 37ºC. When the cells were detached from the flask, inactivation medium (RPMI 1640 with 10% FBSi) was added. Fifty µl of the cell suspension were added to equal volume of Trypan Blue solution, counted in a Neubauer chamber, and the cell density was determined. The volume corresponding to $1x10^6$ cells was centrifuged for 5 minutes at 800 rpm, the cell suspension was ressuspended in BEGM medium and then transferred to a new culture flask and incubated.

The A549 cell line was obtained from the American Type Culture Collection (ATCC No. CCL-185; see Figure 4); it was isolated from a lung carcinoma of a 58 year old Caucasian male. These cells display adherence properties, and are suitable transfection hosts [53].

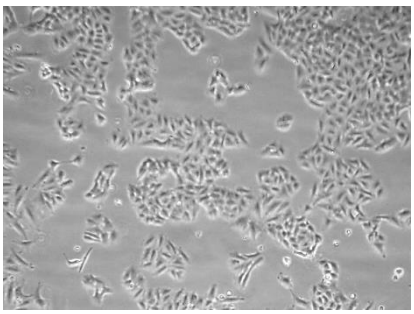


Figure 4. A549 cells in DMEM culture medium.

These cells possess important molecules involved in the detoxification of the cells, such as P450 cytocrome. For this reason, the A549 cell line is important in the study of metabolic pathways, as well as the mechanisms involved in drug delivery and processing at the pulmonary epithelium [55].

The growth medium used for the cell cultures in the first 2/3 passages after defrosting was DMEM, with 1% Pen/Strep, 2.5% HEPES buffer and 10% heat-inactivated Hyclone FBS. Later, when the cells stabilized in their normal pattern of growth, this medium was supplemented with 1% fungizone. The cells were maintained in culture flasks in an incubator, at 37 ºC, in 5% CO_2.

When the cells reached 80% confluence, a subculture was performed: the cells were washed with preheated trypsin-EDTA, and incubated for 4 minutes in an incubator at 37ºC with trypsin-EDTA. When the cells were detached from the flask, culture medium was added to the cell suspension to inactivate the trypsin-EDTA; the suspension was then divided to new culture flasks, depending on the growth rate of the cells before the trypsinization process, and incubated in the same conditions as before.

2. Nanomaterials

In this project, three different MWCNT were studied: NM-400, NM-401 and NM-402 from the Joint Research Centre Repository (Institute for Health and Consumer Protection, European Commission, Ispra, Italy). Previously, a different project with similar goals as this one ("NANOGENOTOX – Safety Evaluation of Manufactured Nanomaterials by Characterization of their Potential Genotoxic Hazard"; Grant agreement 2009 2101) tested NM-402 and NM403 (another MWCNT with comparable features to the three mentioned above) in human lymphocytes and *in vitro* models of human respiratory tract tissues cells (BEAS-2B and A549 cell lines). The characteristics of NM-403 were added to this section and the conclusions

obtained in the mentioned work were added to the analysis of the results, in order to compare them to those obtained in this project.

All of these nanomaterials have applications in the energy industry and are commonly used as structural composites. In terms of impurities, a small percent has been found in these nanomaterials, particularly aluminum, iron and zinc [56, 57]. Their properties differ in several aspects, especially in size; the most relevant characteristics may be consulted in Table 3.

Table 3. General characteristics of the studied nanoparticles [32]. * – [57]

Nanomaterial	Average Length ± SD (nm)	Average Diameter/Thickness ± SD (nm)	Surface Area (m^2/g)	Aspect Ratio ± SD
NM-400	726.3 ± 1.8	10.8 ± 1.3	280	67.3 ± 1.8
NM-401	3366.4 ± 1.9	62.8 ± 1.4	300	53.6 ± 2.0
NM-402	1141.3 ± 2.0	10.7 ± 1.3	250	107.1 ± 1.9
NM-403	394.3 ± 1.6	11.1 ± 1.5	135*	35.6 ± 1.8

NM-402 and NM-403 have already been tested for their genotoxicity in the A549 cell line and in human lymphocytes. The results were ambiguous, pointing to toxicity in some cases and not in others: in A549 cells, a decrease in cell viability was noted in both nanomaterials, but a genotoxic effect (coupled with a dose-response relationship) was only proved in NM-402 and in one of the performed genotoxicity assays (Micronucleus assay); in human lymphocytes, on the other hand, no cytotoxicity was observed in either case, but both nanomaterials have shown genotoxic effects (without a dose-response association). NM-403 was also tested in BEAS-2B, and the results proved that this nanomaterial is not cytotoxic or genotoxic in this cell line.

In that project, it was not possible to make any association between the dimensions of the nanomaterials and their toxicity; consequently, other explanations for those results were proposed, such as surface properties or impurities in the solution [32]. This last point is in agreement with the findings of other authors, who suggested that metal traces may be responsible for the biological effects nanomaterial solutions have in cells [58].

The other two nanomaterials (NM-400 and NM-401) have been tested only in human lymphocytes, showing negative results for genotoxicity [32]. This data is summed in Table 4.

Table 4. Results obtained in previous genotoxicity assays using the four mentioned nanomaterials. **-** negative results, **+** positive results, **+/-** positive results is some assays, and negative in others.

Nanomaterial	Lymphocytes		A549		BEAS-2B	
	Cytotoxicity	Genotoxicity	Cytotoxicity	Genotoxicity	Cytotoxicity	Genotoxicity
NM-400	-	-				
NM-401	-	-				
NM-402	-	+	+	+/-		
NM-403	-	+	+	-	-	-

a. Nanomaterial Sample Preparation

For the dispersion of the MWCNT, an adaptation of the protocol used in the project NANOGENOTOX was used [59]. The nanomaterials were weighed in a precision scale, inside a glass scintillation vial. Then, the powder was prewetted with 96% ethanol (0.5% of the volume of the final solution), and diluted in sterile 0.05% w/v Bovine Serum Albumin (BSA; 99.5% of the volume of the final solution; from Sigma-Aldrich, St. Louis, MO, USA), in order to obtain a stock solution with the final concentration of 2.56 mg/mL (1.28 mg/cm^2).

The concentration of BSA to be used was determined by viscosity, size and dispersibility analysis of the dispersion [56]. Since previous studies indicated that none of the tested nanomaterials presented significant cytotoxicity, the stock nanomaterial concentration was chosen based on the dispersibility of the particles (the maximum amount of nanomaterial that could disperse homogenously in BSA water 0.05%) [32].

The scintillation vial with the nanomaterial dispersion was placed in a container with ice to avoid overheating, and was sonicated for 16 minutes at 400 W and 10% amplitude using a Branson Sonifier S-450D and a 13 mm probe (Branson Ultrasonics Corporation, Danbury, USA). All the treatment solutions were prepared by successive dilutions of the nanomaterial stock dispersion in sterile 0.05% BSA-water, and then by diluting the resulting solutions in culture medium in order to obtain the different concentrations used: 16, 32, 64 and 128 µg/cm^2. The highest concentration was chosen based on the percentage of the batch dispersion (10%) that could be added to a cell culture without interfering with the normal cellular activity and proliferation capacity [32, 59].

b. Scanning Electron Microscopy

Several different samples were prepared with the objective of being observed on a scanning electron microscope (SEM). A549 cells were plated on a 24-well plate at a density of 2x10^5 cells/well and allowed to

grow for approximately 24 hours, at 37 ºC, in 5% CO_2. Then, the cells were treated with NM-401 in only the highest concentration tested – 128 μg/cm^2 – for 18-20 hours, and incubated as before. After this exposure period, the cells were washed with PBS, detached from the plate with trypsin-EDTA and centrifuged for 5 minutes at 800 rpm. The samples were put on custom-made pins and allowed to dry before being inserted on the microscope and observed.

3. Viability

Cell proliferation and viability are important parameters in the assessment of any particle's toxicity. Cytotoxicity, or the ability to reduce the number of viable cells, is a common trait of a lot of different particles, including nanomaterials; for that reason, cytotoxicity and viability assays are usually the first tests performed when analyzing a given chemical or particle [60].

The Giemsa and the methanol are from Merck (Darmstadt, Germany). Mitomycin C is from Sigma-Aldrich (St. Louis, MO, USA). The Gurr's phosphate buffer is from VWR (Radnor, PA, USA).

a. Trypan Blue Exclusion Assay/Cell Counting Method

Trypan blue is a dye that, when added to a cell suspension, enter the cells which membranes have been compromised (or, generally, the non-viable cells) and make them blue, leaving the viable (not damaged) cells in their original color, undyed. Using a Neubauer chamber, it is then possible to count the number of viable and non-viable cells, allowing the comparison with a control sample, and the assessment of the cytotoxicity of the tested particle [52, 61]. Even though this assay is simple and quickly performed, it requires manual counting of cells, being prone to errors.

After each exposure to the nanomaterials (3 or 24 hours) or to the ethyl methanesulfonate (1 or 3 hours), an assessment of the cell viability was performed, using Trypan Blue to exclude non-viable cells. A small volume of the cell suspension was diluted 1:1 in Trypan Blue dye, placed in a Neubauer chamber, and counted. The result was then doubled, to compensate the previous dilution with the dye and the cell concentration times 10^4 (cells/ml) was obtained. In general, few or no unviable cells were detected using these exposure times. Thus, the concentration of viable cells in the exposed wells was then compared to the concentration of viable cells in the negative control well, and the percentage of viability was determined, relatively to the control.

b. Clonogenic Assay

Two of the most common features of cell death are the loss of reproductive integrity and the inability to proliferate. These are the endpoints of the clonogenic assay. It stands on the principle that a cell that retains the capacity to divide is, therefore, able to form a colony; by comparing the number of cells initially plated with the number of colonies formed after an incubation period, the toxicity of the agent can be calculated [62, 63].

This assay could not be performed in BEAS-2B cells, since the reduced number of cells necessary for the formation of individual colonies does not allow these cells to grow.

The A549 cells were plated in a very low density – approximately 250 cells per plate well, in a 6-well plate – and allowed to attach for 20 hours; then, the cells were exposed to the nanomaterials, in the same concentrations as stated above. The attachment period was shorter than the doubling time of the cells – 22 hours – guaranteeing that the cells were attached but not divided at the time of the treatment with the nanomaterials. Mitomycin C (MMC) was used as positive control. The plates were then incubated for 8 days, at 37 ºC, in 5% CO_2.

After the growth period, the cells were washed twice with PBS and fixed with absolute cold methanol for 10 minutes. After a drying period, the colonies were stained with Giemsa (10%) for 10 minutes, washed twice with Gurr's phosphate buffer and allowed to dry. The colonies were counted, and several parameters were calculated in order to compare the nanomaterial-treated cultures to the ones of the control sample, using the following equations [63]:

$$\text{Plating Efficiency} = \frac{\text{number of colonies in the negative control}}{\text{number of cells plated in each well}} \quad \text{(equation 1)}$$

$$\text{Surviving Fraction} = \frac{\text{number of colonies exposed to the treatment}}{\text{number of colonies in the negative control}} \quad \text{(equation 2)}$$

$$\text{Cytotoxicity} = 100 - (\text{Surviving Fraction} \times 100) \quad \text{(equation 3)}$$

From the equations obtained in the regression analysis performed on these results, when possible, the half maximal inhibitory concentration (IC50) was also calculated.

c. Proliferation and Replication indexes

The viability of the cells exposed to the carbon nanotubes was also explored with the cytokinesis-blocked proliferation index and the replication index, calculated in the analysis of the results of the Micronucleus assay, as is explained below.

4. Genotoxicity

As mentioned above, genotoxicity refers to the capacity of an agent to cause damage to DNA, either directly or indirectly; this can ultimately lead to cell death or, alternatively, to cancer. Some of the most common aggressions to the genome of cells are breaks in the DNA sequence, and structural or numeric alterations to the chromosomes, which can cause alterations in gene expression. Mutations in some of the genes essential for the maintenance of the good functioning of the DNA processes can lead to carcinogenesis: oncogenes and tumor suppressor genes, for example, are critical in the preservation of the genome health, and an agent that causes mutations in them may be associated to an increased risk of cancer. Indeed, the accumulation of genomic errors and, consequently, the acquisition of genomic instability are known events in the beginning of cancer development [64].

The Ethyl Methanesulfonate (EMS), the low melting point agarose, the dimethyl sulfoxide (DMSO), the Triton-X100, the HEPES, the Trizma-base and the ethidium bromide, as well as the cytochalasin-B and the acridine orange are all from Sigma-Aldrich (St. Louis, MO, USA). The normal melting point agarose is from Amersham Biosciences (Uppsala, Sweden), the $Na_2EDTA.2H_2O$ is from Calbiochem (Darmstadt, Germany), and the Tris-HCl is from Invitrogen (Carlsbad, CA, USA). The NaCl, KCl, acid EDTA, NaOH, Entellan, as well as KH_2PO_4 and Na_2HPO_4 are from Merck (Darmstadt, Germany). The Saline-Sodium Citrate Buffer (SSC) is from Gibco (Scotland, UK).

a. Comet Assay

The Comet Assay, or Single Cell Gel Electrophoresis Assay, is a technique that allows the detection of DNA damage in individual cells. The cells, after being trapped on a microscope slide in an agarose gel and lysed (to remove all cellular components except for the nucleus), are subjected to an electrophoresis under alkaline conditions (pH>13). DNA fragments, resulting from single or double strand breaks, being smaller than the molecule itself, have the capacity to migrate more rapidly in the gel towards the anode. After staining the slide with a DNA-binding fluorescent dye (e.g. ethidium bromide) and analyzing the results on a fluorescence microscope, it is possible to identify a distinct comet shape, the head being the largely undamaged DNA molecule, and the tail the trail of broken DNA fragments (presented in Figure 5). Therefore, the length of the comet tail is directly related to the extent of the damage to the DNA [65-69].

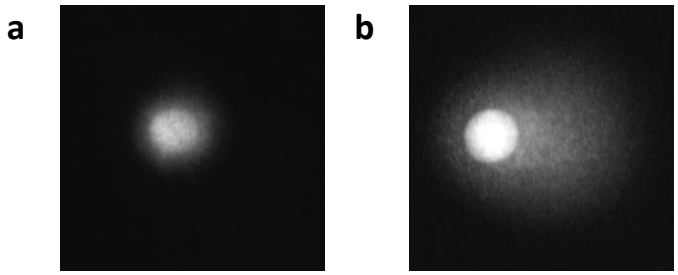


Figure 5. Examples of nucleoids obtained using Comet Assay.
a – undamaged DNA, **b** – high level of DNA damage.

Despite its already high sensitivity, there is a method that can be applied in order to increase the sensitivity of the assay even further. The digestion with enzymes that recognize DNA damage and replaces the error with a break is the most prominent. The use of different enzymes allows the detection of different kinds of damage (such as ultraviolet-induced pyrimidine dimers and oxidized bases), making this modification very useful for different endpoints. One of the most commonly used enzymes in this assay is the formamidopyrimidine DNA glycosylase (FPG), which is a bacterial enzyme that repairs DNA damage and errors. It recognizes oxidized purines (such as 8-Oxoguanine), replacing them with strand breaks. This process increases the tail intensity and thus, the difference between the percentage of DNA in the tail of the nucleoids from unmodified and modified comets show the level of oxidative damage to the DNA [66-69]. In this project, every Comet assay was performed with and without this modification, simultaneously, in order to assess the number of oxidative lesions in each case.

The comet assay has multiple advantages, such as being sensitive, flexible, economic (both in time and in materials) and fast. Therefore, this is a widely used method for the analysis of genotoxicity, both in clinical and environmental studies [68, 69].

The cells were plated at the density of 0.5 x 10^6 cells per well and allowed to grow for 24 or 48 hours (in the case of A549 or BEAS-2B, respectively). Then, the cells were exposed to the concentrations of nanomaterial described in the previous section. The cells were exposed to the treatments for 3 and 24 hours. The positive control was EMS diluted first in PBS and then in culture medium, in the concentration of 0.75 mM. Later on, other concentrations of EMS and different exposure times were tested, in order to find a more suitable positive control response: 0.75, 5, 10, 20, 30 and 40 mM for 1 hour, and 1, 2, 5 and 10 mM for 3 hours; the only change from the protocol described below is that these experiments were carried out on 24-well plates, where the cells were plated in the density of 0.1x10^6 cells/mL.

After the respective exposure time to the treatments, the cells were washed and detached using trypsin-EDTA (see above). The cells were removed from the plate, collected in centrifuge tubes, counted and centrifuged, and (if possible or necessary) the cell density was adjusted to 4x10^6 cells/mL with the

addition of PBS. The cell suspension was then mixed with 0.8% low melting point agarose, placed on microscope slides previously coated in 1% normal melting point agarose and covered with cover slips.

The slides were allowed to dry and the agarose to solidify on a refrigerated surface. Then, the cover slips were carefully removed, and the slides were immersed in lysis solution [freshly prepared before use, with 89% Lysis Buffer (NaCl 2.5 M, $Na_2EDTA.2H_2O$ 100 mM, Tris-HCl 10 mM; NaOH until pH=10), 10% DMSO and 1% Triton-X100] in a coplin jar covered with aluminum foil for approximately 1 hour at 4 ºC.

The slides were washed three times for five minutes each in F buffer (HEPES 40 mM, KCl 100 mM, acid EDTA 0.5 mM, BSA 0.2 mg/mL; KOH until pH=8). Afterwards, FPG enzyme (kindly provided by Dr. A. R. Collins, University of Olso, Norway) diluted in F buffer, or F buffer only was added to each microgel and covered with cover slips, and the slides were placed in a humidified atmosphere in an incubator (37 ºC) for 30 minutes.

The cover slips were then removed, and the slides were covered in electrophoresis buffer (NaOH 0.3 M, $Na_2EDTA.2H_2O$ 1mM; pH=13) for 30 minutes, allowing the DNA to unwind. After this time, electrophoresis was performed for 25 minutes at 28 V and 300 mA.

After electrophoresis, the slides were washed for 10 minutes at 4 ºC twice: first with neutralization buffer (Trizma-base 0.4 M in water, with 9,5 %vol HCl 4 M; pH=7.5), and then with MilliQ water. The slides were stored in an aluminum foil covered box, to dry at room temperature, overnight.

The slides were stained with ethidium bromide (12.5 µg/mL), and observed in a fluorescence microscope (Axioplan2 Imaging, Zeiss), with the assistance of specific image-analysis software (Comet Imager 2.2, from MetaSystems, GmbH). Two slides were prepared for each treatment condition. 50 nucleoids were analyzed per microgel, therefore, 100 per slide, and 200 per treatment.

The median value of the percentage of DNA in the tail was calculated, and used in the statistical analysis of the results. The percentage of DNA in the tail of the nucleoids from FPG-treated cultures ($\%DNA_{FPG}$, in equation 4) was compared to the percentage of DNA in the tail of the nucleoids from untreated cultures ($\%DNA_{untreated}$, in equation 4), and the oxidative damage was calculated using the following equation [68]:

$$\text{Oxidative Damage} = \%\text{DNA}_{\text{FPG}} - \%\text{DNA}_{\text{untreated}} \qquad \text{(equation 4)}$$

b. Cytokinesis-blocked Micronucleus Assay

One of the most important events in tumorigenesis is the occurrence of genetic abnormalities – either resulting from direct damage on the DNA, or from chromosome loss or fragmentation [70]. A common genetic abnormality is micronuclei: whole chromosomes or chromosomal fragments stay behind

during nuclear division, and therefore are not incorporated into the nucleus of the daughter cells; a membrane is formed around them, and they remain in the cytoplasm through future cell cycles [70]. Micronuclei, therefore, as well as other nuclear aberrations, are a sign of chromosomal breakage or mitotic spindle disruption, as well as unrepaired DNA breaks, defective separation of chromatids or even DNA amplification. For this reason, micronuclei are considered a very important cancer biomarker, due to the fact that an increase in micronuclei number is strongly associated to an increase in cancer risk [49, 64, 70].

The best *in vivo* or *in vitro* method to analyze and quantify micronuclei is the Cytokinesis-blocked Micronucleus Assay. In this assay, the cells are allowed to go through one division cycle, before cytochalasin-B is employed to block cytokinesis. For this reason, the majority of the cells at this point are binucleated, having one or more micronuclei where chromosomal breakages or losses have occurred [65].

This assay can also be used to measure other endpoints associated with chromosomal damage – some of them are presented in Figure 6. Nucleoplasmic bridges are formed due to the exposure to clastogens, and originate when a dicentric chromosome (formed due to DNA damage or misrepair) has its centromeres pulled apart to opposite poles of the cell, forming a link between two nuclei. Nuclear buds are micronuclei that are not yet fully separated from the nucleus, and result from the elimination of amplified DNA or DNA-repair complexes. Necrotic cells are also observable using this assay, being easily identified by the presence of multiple vacuoles in the cytoplasm and nucleus, and damaged membranes. Apoptotic cells, on the other hand, have intact membranes, but exhibit nuclear fragmentation [71].

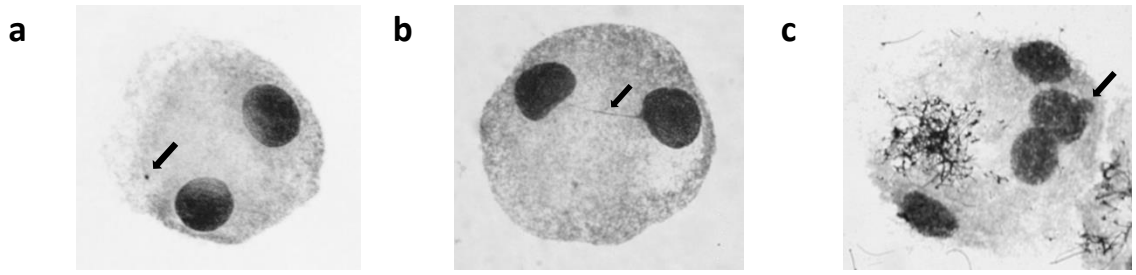


Figure 6. Examples of chromosomal damage in A549 cells (black arrows). Section **a** shows a binucleated cell with a micronucleus, section **b** shows a binucleated cell with a nucleoplasmic bridge connecting the two nuclei, and section **c** shows a multinucleated cell with a nuclear bud (the black fibers are NM401).

Being very reliable and reproducible, this assay is widely used in the evaluation of DNA damage and chromosomal breakage or loss. Allowing the analysis of other chromosome damage markers, it also allows the measurement of other genotoxic and cytotoxic events, such as chromosome rearrangement, cell death and cell replication and proliferation effects [71].

i. BEAS-2B cell line

The BEAS-2B cells were seeded in 6-well plates at a density of 0.5 x 10^6 cells per well, and incubated for 48h at 37 ºC and 5% CO_2. Then, they were exposed to the nanomaterials in the previously referred concentrations, and incubated again in the same conditions. MMC was used as a positive control, and was prepared in PBS and in culture medium, in a final concentration of 0.15 µg/mL with the same exposure time. After six hours of exposure, cytochalasin-B was added to each well, in a final concentration of 9 µg/mL.

Forty-eight hours after the treatment, the cells were washed with pre-heated PBS and detached with trypsin-EDTA in the same manner as described above. Then, they were centrifuged (all the centrifugations in this method are carried out for 5 minutes at 1200 rpm), and the cell pellet was ressuspended in PBS. After a second centrifugation, the cells were submitted to a hypotonic shock with a RPMI 1640:MilliQ water (1:1) solution, added vigorously. The cells were added fix solutions twice: first, with a refrigerated solution of 3 parts methanol to 1 part acetic acid, and later with a 97% methanol, 3% acetic acid solution. Finally, after centrifugation, the cell pellet was ressuspended and two drops were placed directly on a microscope slide, from a height of ~2 cm; if necessary, the cell density was adjusted with the addition of PBS after careful observation on a microscope. For each treatment, two to four slides were prepared.

The slides were allowed to dry for a few days before staining. The solutions used in the staining process were: Sörensen buffer (KH_2PO_4 66.72 mM and Na_2HPO_4 66.71 mM), SSC 2x, acridine orange (stock solution = 32 µg/mL) and DAPI. The slides were immersed in acridine orange solution (1:30 in Sörensen buffer, pH 6.8), and washed three times in Sörensen buffer. Then, a few drops of DAPI (5 µg/mL in 2xSSC) were added to the slides, and cover slips were used to cover them. After five minutes, the cover slips were removed and the slides were washed under running tap water. The slides were allowed to air-dry, and were then stored in an aluminum foil covered box in 4 ºC.

ii. A549 cell line

The A549 cells were seeded in 6-well plates at the same density as previously reported – 0.5 x 10^6 cells per well; however, the incubation at 37 ºC and 5% CO_2 lasted only 24 hours instead of 48h used in the case of BEAS-2B. The cells were exposed to the nanomaterials in the previously referred concentrations, and incubated again in the same conditions. The positive control was the same as above (MMC) which was prepared in PBS and in normal culture medium (final concentration of 0.15 µg/mL). After six hours of exposure, cytochalasin-B was added to each well, in a final concentration of 6 µg/mL.

Forty-eight hours after the treatment, the cells were washed with pre-heated PBS and detached with trypsin-EDTA in the same manner as described above for this cell line. The suspension was centrifuged for 5 minutes at 1000 rpm, and the cell pellet was ressuspended in culture medium. The cells were then

submitted to a hypotonic shock with a solution of 73.5% sterile bidistilled water + 24.5% culture medium + 2% FBSi, added drop by drop under agitation. The cells were centrifuged for 5 minutes at 1000 rpm; the cell pellet was ressuspended and two drops were placed directly on a microscope slide, from a height of ~2 cm. If necessary, the cell density was adjusted with the addition of PBS after careful observation on a microscope. For each treatment, two/four slides were prepared.

The slides were allowed to dry for a few hours and were then immersed in a refrigerated solution of 3 parts methanol to 1 part acetic acid for 20 minutes to fix the cells. After drying for a few days, the slides were stained with Giemsa. The slides were immersed in Gurr's phosphate buffer for 4 minutes, then in a solution with 4% Giemsa (in Gurr's phosphate buffer) for 15 minutes, and washed twice with the same buffer. After air drying, the slides were mounted with Entellan and cover slips.

iii. *Analysis*

In all assays, 500 binucleated cells were analyzed per slide, therefore, 1000 per culture, and 2000 per treatment.

The criteria used in this project to score binucleated cells has been extensively characterized by Fenech [71], and therefore shall not be described in detail. The general points are: the cells must have two nuclei; the nuclei may touch and a fine nucleoplasmic bridge may be formed between them, as long as the definition of the nuclei is not lost; the nuclei must have intact membranes and be clearly located within the same cytoplasmic area; the size and staining characteristics of the nuclei must be approximately identical.

The criteria for scoring micronuclei was defined by the same author [71], and may be summarized by the following points: the diameter of the micronuclei must be less than 1/3 the diameter of the main nuclei, and must have a round or oval shape; micronuclei are not connected to the main nuclei, and must not overlap it (both boundaries must be clearly distinguishable); the staining characteristics of the micronuclei must be approximately identical to those of the main nuclei.

Based on the analysis of the binucleated cells alone, the mean number of micronuclei per 1000 binucleated cells was obtained. Based on the number of mononucleated, binucleated and multinucleated cells found in each treatment, the Cytokinesis-blocked Proliferation Index (CBPI) and the Replication Index (RI) were calculated. The equations used were the following [32]:

$$\mathrm{CBPI} = \frac{\mathrm{N^{O}\ Mononucleated\ cells} \ + \ 2\ \mathrm{x\ N^{O}\ Binucleated\ cells} \ + \ 3\ \mathrm{x\ N^{O}\ Multinucleated\ cells}}{\mathrm{Total\ number\ of\ viable\ cells}} \qquad \text{(equation 5)}$$

$$\mathrm{RI} = \frac{\left(\frac{\mathrm{N^{O}\ Binucleated\ cells} \ + \ 2\ \mathrm{x\ N^{O}\ Multinucleated\ cells}}{\mathrm{Total\ number\ of\ cells}}\right)_{\mathrm{Treated\ cultures}}}{\left(\frac{\mathrm{N^{O}\ Binucleated\ cells} \ + \ 2\ \mathrm{x\ N^{O}\ Multinucleated\ cells}}{\mathrm{Total\ number\ of\ cells}}\right)_{\mathrm{Control\ cultures}}} \qquad \text{(equation 6)}$$

5. Statistical Analysis

The general processing of the data was performed in Microsoft Office Excel. The statistical analysis of the results was performed in IBM SPSS Statistics 22.

Both the viability results from the cell counting method and the results from the clonogenic assay – plating efficiency, surviving fraction and cytotoxicity – were analyzed using the Student's *t*-test, comparing the percentage of viability of nanomaterial exposed cultures to the control cultures. The proliferation and the replication indexes (CBPI and RI, respectively) were analyzed using Student's *t*-test or One-Way ANOVA test.

The results of the Comet Assay of the nanomaterial exposed cultures were compared to those of the control cultures using the One-Way ANOVA test, if the results were assumed to follow a normal distribution, or the non-parametric Kruskal-Wallis Test, if that was not the case.

In the Micronucleus Assay, the Two-sided Fisher's exact test was applied to compare the frequency of micronucleated binucleated cells between nanomaterial exposed cultures and control cultures.

When relevant, the possibility of the existence of a dose-response curve was evaluated using regression analysis; this step proposes the best mathematical model that predicts the behavior of the results (by means of a curve equation) and offers a correlation coefficient (R^2), which indicates how well this proposed model relates to the data.

Results

1. Qualitative Analysis of the Dispersion of the tested Nanomaterials

As mentioned before, the protocol used for the dispersion of the CNT in this study was a standard procedure that was developed in NANOGENOTOX, with the aim of ensuring the stable dispersion of the nanomaterials, reducing agglomeration/aggregation and subsequent sedimentation [59]. It has been described as a suitable protocol for the majority of the nanomaterials tested in that project, and therefore, it was considered suitable to be used in this case too. However, since the beginning of this work, some issues with the dispersion of the CNT have been noted, especially in terms of the maintenance of the homogeneity of the dispersion over time. For that reason, the differences observed in the nanomaterial suspension throughout the duration of the experiments were photographed and are documented here.

In Figure 7, it is possible to observe the quality of the nanomaterial dispersion in BSA-water inside the scintillation vials used for the sonication of the particles. The comparison of the photographs displayed in each column shows the aspect of nanomaterials before, immediately and 30 minutes after the dispersion procedures. If the comparison is made along each column, the differences in the images correspond to different characteristics of the nanomaterials, since each row contains photographs of a different CNT, although all are MWCNTs. It is noticeable that the nanomaterials seem to be well dispersed immediately after the sonication, even though a portion of particles are attached to the glass container. However, after 30 minutes (the time needed for the preparation of the treatment solutions and their addition to the cell cultures), a large portion of the particles remained attached to the scintillation vial walls. It is important to note that the aspect of the particles that stayed attached to the vial walls is very distinct between the different nanomaterials: NM-400 seems to form large clusters that remain agglomerated and stably attached to the glass even after vigorous agitation of the solution; NM-401, on the other hand, forms smaller masses that did not seem to attach so firmly to the glass wall, as a mild agitation easily removed them; in the case of the NM-402, the particles attached to the walls are smaller and seemingly more individualized, although in greater amounts than in the previous nanomaterial; NM-403 is quite well dispersed, without clusters attached to the glass walls, and the dispersion has an homogenous appearance.

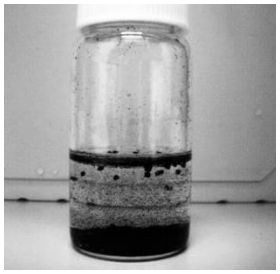
	Before sonication	Time after sonication (minutes)	
		0	30
NM-400			
NM-401			
NM-402			
NM-403			Not Performed

Figure 7. Scintillation vials containing the studied CNT.

In Figure 8, the differences in aspect between the slide preparation of cells exposed to the three nanomaterials are also apparent. NM-400 (section **a**), as well as NM-402 (section **c**) have a seemingly granulated aspect, with very small particles filling the space between the cells. NM-401 (section **b**), on the other hand, has much longer and clearly distinguishable fibers.

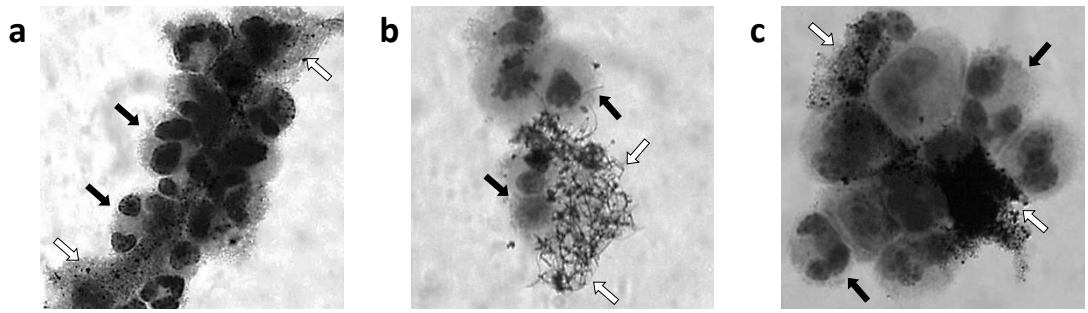


Figure 8. The three studied nanomaterials over or attached to Giemsa-stained BEAS-2B cells (1000x magnification): **a** – NM-400; **b** – NM-401; **c** – NM-402. The black arrows point to cells, the white arrows point to nanomaterials.

In the specific case of NM-401 (due to the fact that it was the most clearly visible under the microscope, since it is the longest and thickest one), a more thorough analysis of the quality of the dispersion was performed; this is documented in Figure 9. In section **a**, where the CNT solution was not sonicated and only had been manually agitated, it is possible to note that before sonication, there are many large agglomerates, and practically no individual particles; in section **b**, on the other hand, it becomes apparent that after sonication, the number of large agglomerates decreased considerably, and the amount on individualized fibers increased greatly. This demonstrates that the dispersion procedure was successful, and that the dispersion of the nanomaterial was achieved. Section **c** presents the aspect of the stock solution after 30 minutes (the time needed to treat the cells with the nanomaterial, as stated above): agglomerates and aggregates are starting to form, but a high number of single fibers in the solution can still be seen. Section **d** represents the nanomaterial stock solution diluted 1:10 in DMEM culture medium, in the concentration of 128 $\mu g/cm^2$, as would be directly used as the top concentration for cell treatment. Small sized particles are seen, although many agglomerates are still observable.

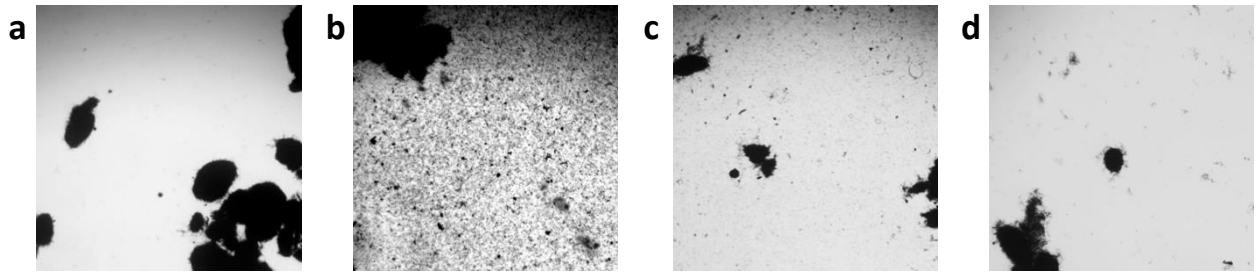


Figure 9. NM-401 in different dispersion states (100x magnification): **a** – before sonication, just mildly agitated; **b** – immediately after sonication; **c** –30 minutes after sonication; **d** – diluted 1:10 in DMEM culture medium.

Figure 10 shows the aspect of the three tested MWCNT in each plate well at the same concentration, immediately after the treatment, three hours later and twenty four hours after treatment. It is important to note that these photographs portray the CNT dispersed in BEGM culture medium.

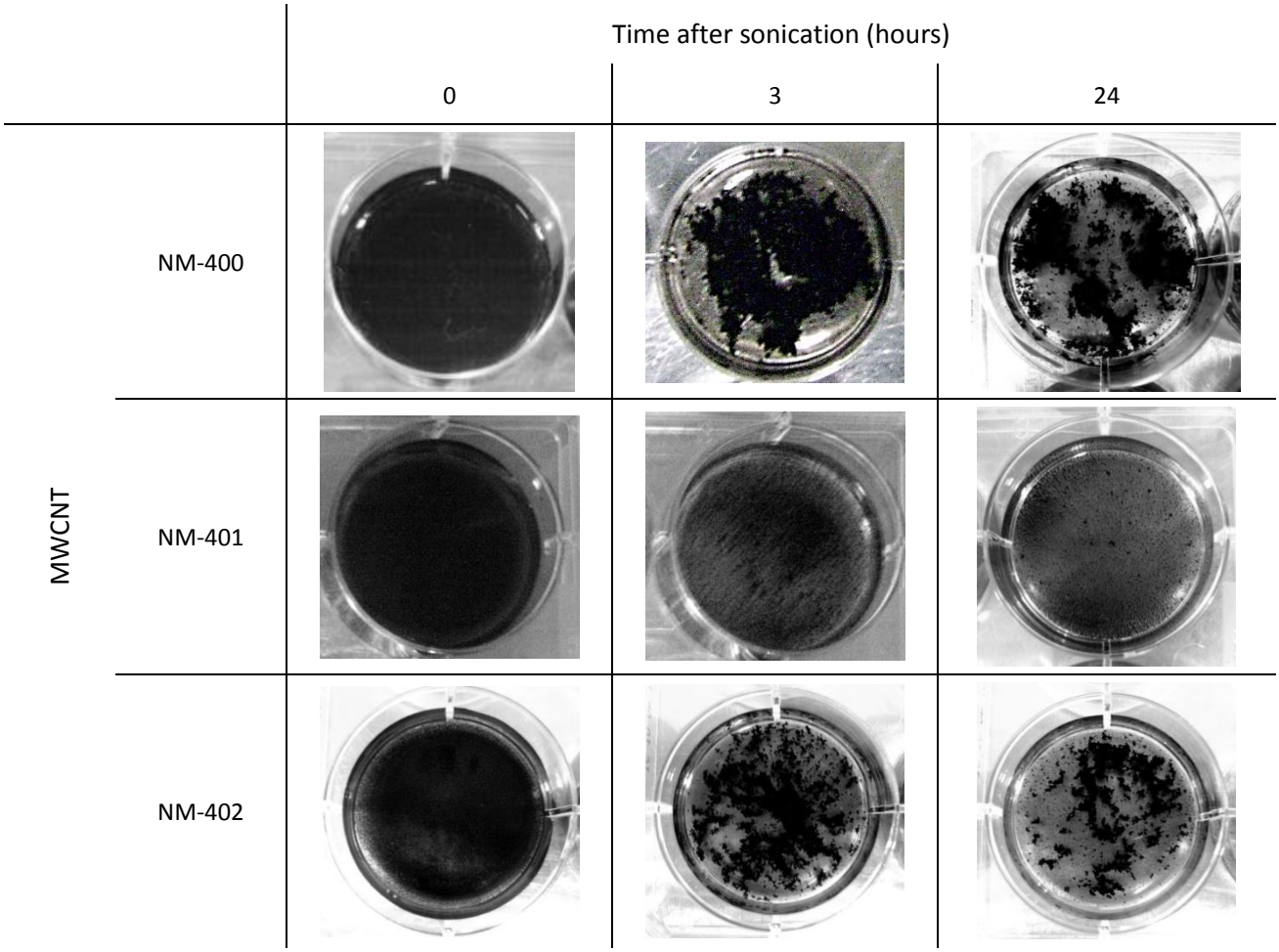


Figure 10. The three MWCNT in suspension in BEGM culture medium, immediately after cell exposure, three hours later and twenty-four hours after treatment. The nanomaterials are in a final concentration in the culture medium of 32 µg/cm^2.

Between the photographs in the first column, the differences are not apparent, since the CNT are seemingly completely dispersed. However, three hours after the treatment, the homogeneity of the dispersion has changed, as masses of nanomaterial can be distinguished, especially in NM-400 and NM-402. The last column shows the further decrease in the homogeneity of the dispersions after 24 hours. Sedimentation is also observed. Figure 11 represents the aspect of a plate well in which the cells were exposed to the same concentration of NM-402 particles as in the previous figure (32 µg/cm^2) for only 15 minutes – therefore, 45 minutes after dispersion. It is observable that the particles are already forming visible agglomerates, even though these are still quite small at naked eye. However, comparing this image to the same concentration 3 hours after treatment in Figure 10, it is possible to note how the aspect of the dispersion did not change much in the following 2 hours and 15 minutes. This suggests that the formation of most of the masses of nanomaterial happens shortly after the dispersion; therefore, in most of the exposure period, it seems that the cells are exposed to large agglomerates of nanomaterials, as well as to the individual particles.

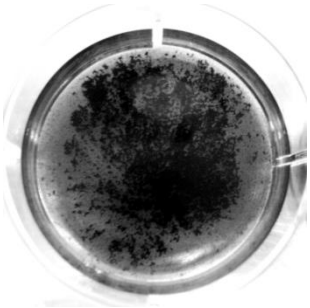


Figure 11. NM-402 suspension in BEGM culture medium, 15 minutes after the treatment (approximately 45 minutes after dispersion). The nanomaterial suspension is in a concentration of 32 µg/cm^2.

Figure 12 represents the lateral aspect of the plate wells; it is possible to note that the majority of the nanomaterial is deposited in the bottom of the well. When the culture medium with the CNT was removed (after the exposure period), a very large amount of the particles was instantly removed as well, and only a small fraction remained attached to the cells.

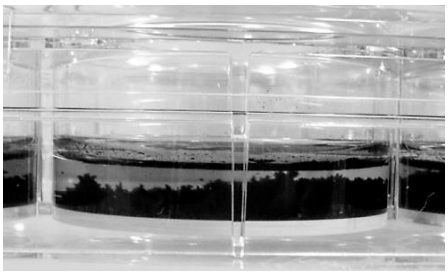


Figure 12. Lateral perspective of a plate well, with NM-402 in BEGM culture medium.

It is noteworthy, though, that the portion of particles that remained attached to the cells increased proportionally to the concentration of CNT, being harder to remove with PBS after the exposure to higher doses. Small portions of NM-401, in particular, were sometimes impossible to remove, which resulted in blackened cell suspensions and microscope slides with visible dark spots; this was possibly due to the considerably smaller size of the agglomerated masses of NM-401 particles in comparison to NM-400 or NM-402.

As mentioned above, scanning electron microscopy was used to visualize A549 cells treated with NM-401. In Figure 13, it is possible to distinguish the cells (evidenced by white arrows with black outline) as small spherical units with a diameter of 12-19 µm, and carbon nanotubes (black arrows with white outline) as long fibers with an approximate length of 5 µm. However, this last number is considered very inaccurate, due to the fact that the software coupled to the electron microscope could not take measurements in that order of values, making visual interpretation the only way to assess the length of the tubes. As it is quite evident in the image, the nanotubes are bent and tangled over each other and the cells, which makes a correct assessment of the length of the nanoparticles difficult.

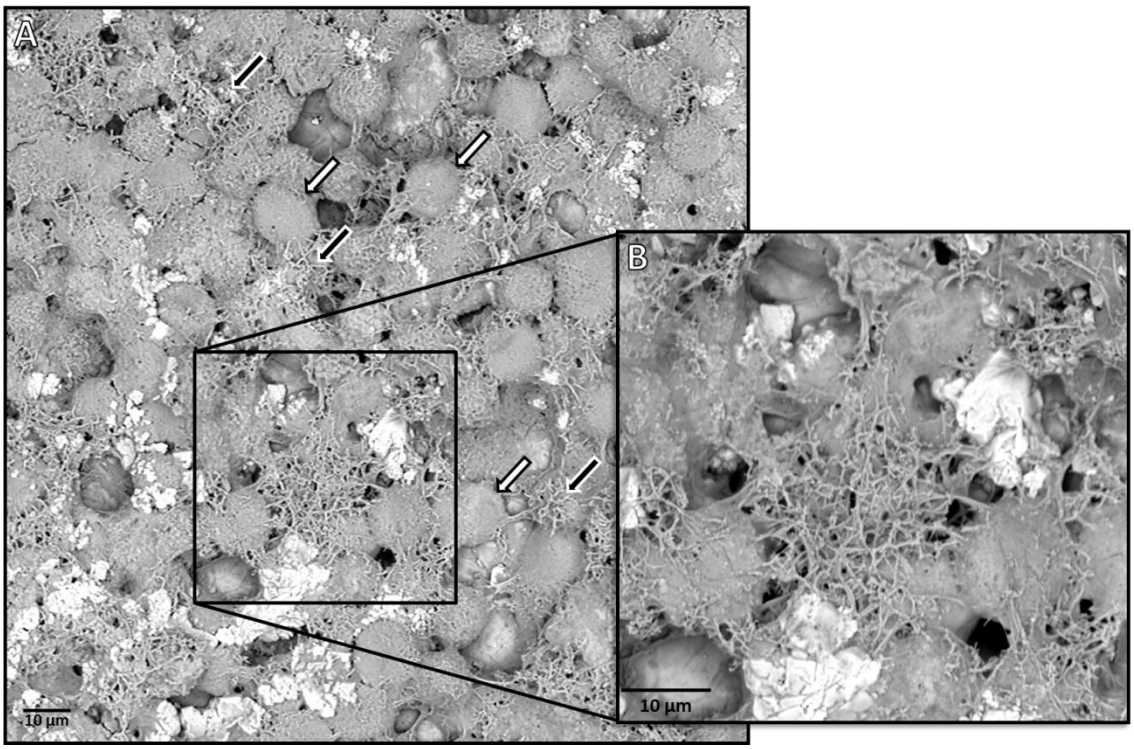


Figure 13. Scanning Electron Microscopy image of A549 cells treated with NM-401. The white arrows with black outline point to cells, and the black arrows with white outline point to visible clusters of nanomaterial. Section **A** represents a large area of the image, while **B** focuses on a smaller one, emphasizing on a mass of nanoparticles. The bright white masses are crystallizations of the salts present in the culture medium when the sample was dried.

2. Tests for the Validation of the Methods – Ethyl Methanesulfonate

a. Viability

In the case of the BEAS-2B cells, it is possible to observe a lack of a strong cytotoxic response when treated with EMS, in Figure 14-a. The viability after the concentration of 30 mM in the shorter exposure time and the concentration of 5 mM in the longer one were significantly different from the negative controls (p<0.01 in both cases; Student's t-test). No dose-response associations were observed.

In the case of A549 cells treated with EMS, it is apparent a similar response as in the previous case, recognizable by the same mild decrease of viability, never reaching a value of 50% (Figure 14-b). However, this experiment revealed more results with significant differences from the control than in the case above: the concentrations of 30 and 40 mM in the 1 hour exposure (p=0.042 and 0.011, respectively), and the concentrations of 5 and 10 mM in the 3 hour exposure (p=0.011 and 0.037, respectively). None of the results has a dose-response association.

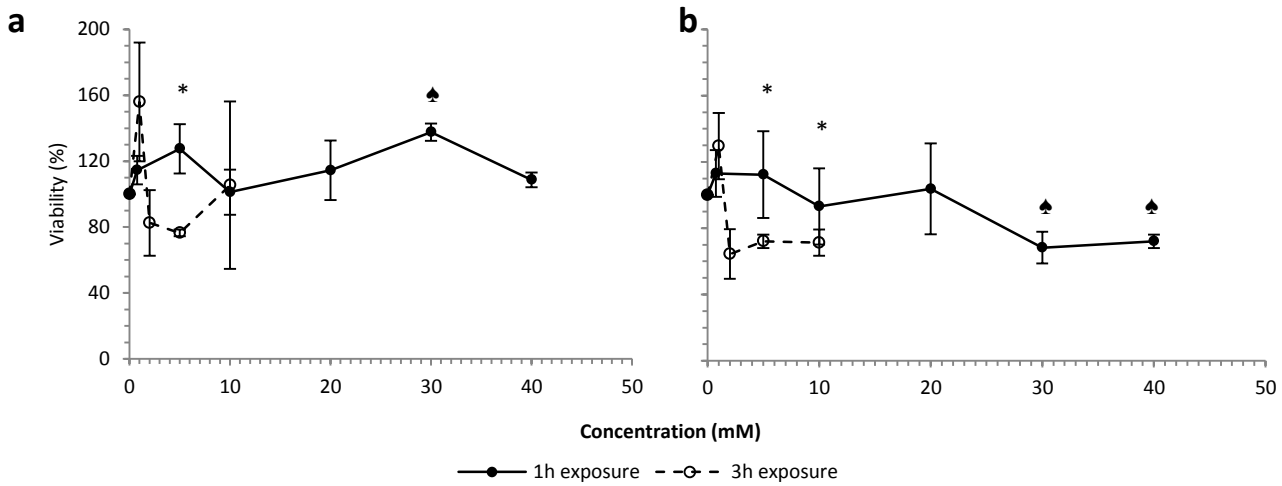


Figure 14. Results of the cell counting following EMS exposure. **a** – BEAS-2B; **b** – A549. Significantly different from the control (p<0.05, Student's t-test): ♠ – 1h exposure; * – 3h exposure.

b. Genotoxicity

As mentioned above, different concentrations of EMS were tested with Comet assay. First, using the conventional Comet assay (without the use of FPG) in BEAS-2B cells, EMS caused a high level of damage on the DNA of the cells following both 1 hour and 3 hour exposure, with several values significantly different from the control: in the first case (1 hour exposure), the concentrations of 10 and 40 mM induced a level of DNA damage significantly different from that of control (p=0.05 and 0.005, respectively; One-Way ANOVA test, with post-hoc); in the second case (3 hour exposure), the effect of the two highest concentrations was significantly different (p=0.036 and 0.030, respectively). These results may be observed in Figure 15.

Regression analysis showed the best fitting model corresponded to a linear relationship for both cases.

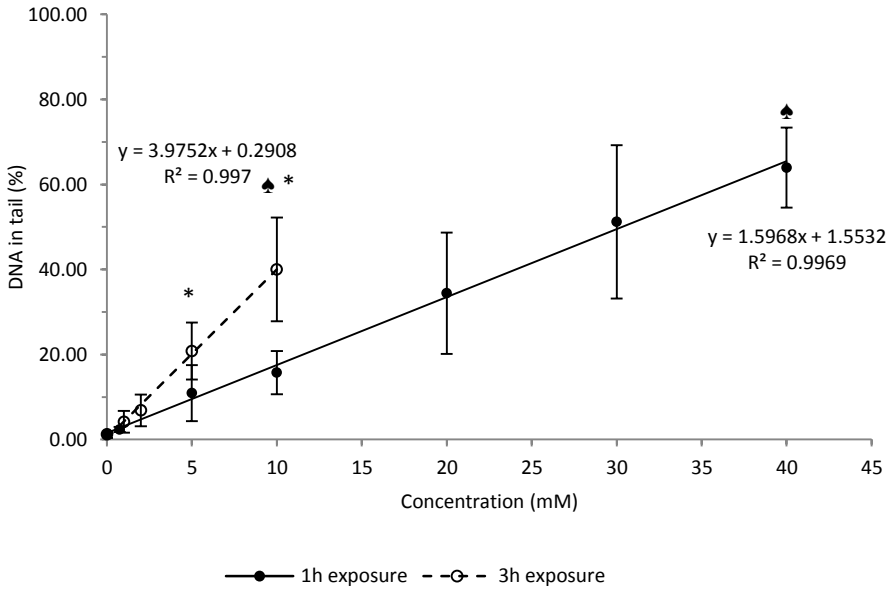


Figure 15. Results of the unmodified Comet Assay with EMS on BEAS-2B cells. Significantly different from the control (p<0.05, One-Way ANOVA test): ♠ – 1h exposure; * – 3h exposure.

In the comet assay modified with FPG, EMS caused a higher level of damage on the DNA of the cells than in the conventional assay, in both 1 hour and 3 hour exposures – Figure 16. Besides, both curves appear to reach a plateau (close to 80% DNA in tail), where the value stabilizes.

After statistical analysis, it is possible to conclude that several values of the % DNA in tail are significantly different from the control: in 1 hour exposure the concentration of 0.75 mM was significantly different from control (p=0.019), as well as the three highest concentrations (p=0.001, 0.002 and 0.001 respectively;); in 3 hour exposure, the concentrations of 1, 5 and 10 mM showed significance (p=0.010 in the first case and p<0.001 in the other two).

Regression analysis showed that, due to the apparent stabilization of the curve, a linear-quadratic association is the best fitting model for both cases.

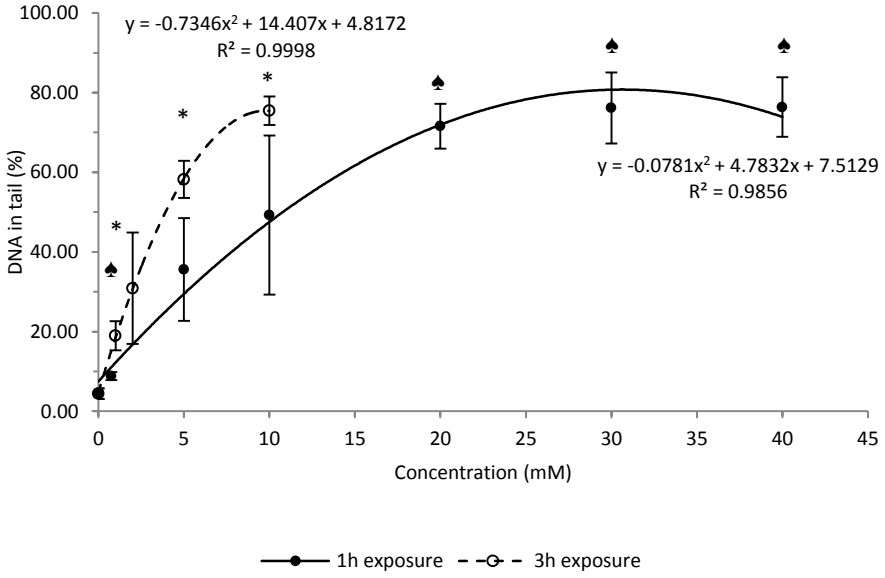


Figure 16. Results of modified Comet Assay with EMS on BEAS-2B cells. Significantly different from the control (p<0.05, One-Way ANOVA test): ♠ – 1h exposure; * – 3h exposure.

The results of oxidative DNA damage calculated as the difference in % DNA in tail between the two assays – the modified and the unmodified - may be observed in Figure 17. As was expected, since the curve from the unmodified assay is consistently linear with a positive slope, and the curve from the modified assay stabilizes after some time, the curve for the oxidative damage resembles a parabola. There is only one value in each exposure time that is significantly different from the control: in the 1 hour exposure, the concentration of 20 mM, and in the 3 hour exposure, the concentration of 5 mM, both have a p=0.031. Regression analysis offers a quadratic model for both of these curves.

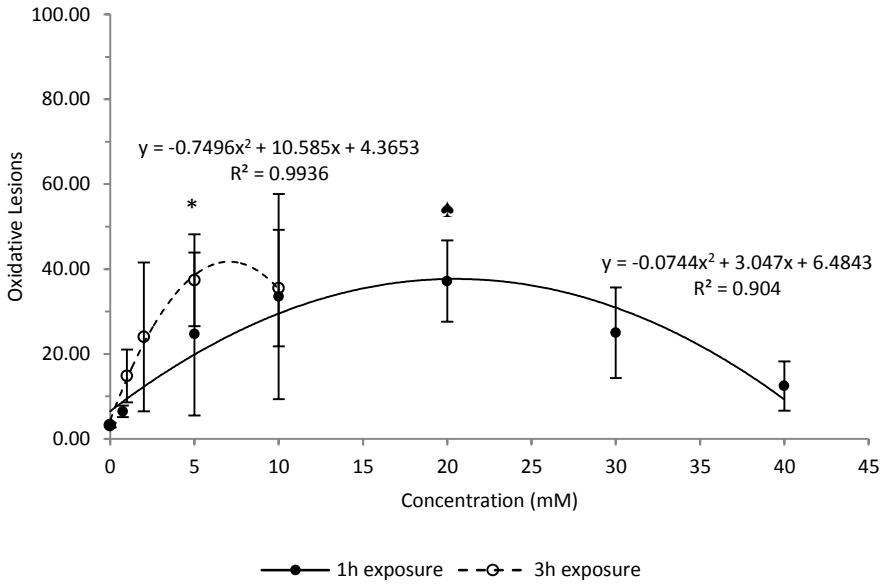


Figure 17. Oxidative damage caused by EMS on BEAS-2B cells. Significantly different from the control (p<0.05, One-Way ANOVA test): ♠ – 1h exposure; * – 3h exposure.

The Comet assay with different doses of EMS was also performed on A549 cells, and the results are consistent with the ones presented above. The comet assay without the use of FPG (Figure 18) showed high level of damage on the DNA of the cells in both exposure times, with several values significantly different from the control at 1h exposure (10, 20, 30 and 40 mM), as well as with 3 hour exposure (2, 5 and 10 mM) (p<0.001 in all of them). Regression analysis showed that, as above, a linear dose-response relationship was found for both cases.

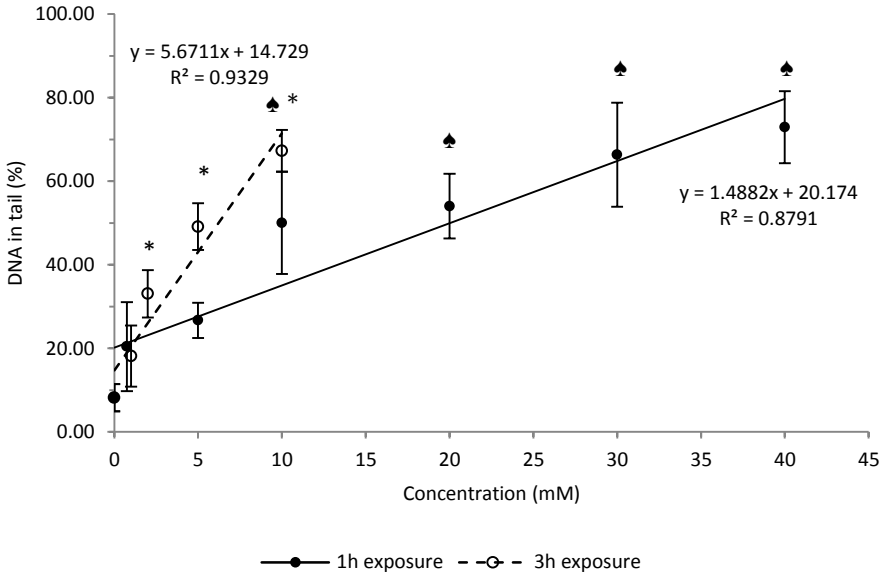


Figure 18. Results of unmodified Comet Assay with EMS on A549 cells. Significantly different from the control (p<0.001, One-Way ANOVA test): ♠ – 1h exposure; * – 3h exposure.

In the comet assay with FPG, as was expected, EMS caused more oxidative damage on the DNA of the cells than when the enzyme was not used, in both 1 hour and 3 hour exposures (Figure 19). Besides, even more evidently than in the same treatment in the case of the BEAS-2B cells, both curves appear to reach a plateau (close to 70% DNA in tail), where the value of percentage of DNA in the comet tail stabilizes. In terms of statistical analysis, all the values are significantly different from the negative control, at both exposure times (p<0.01 in all of them; One-Way ANOVA test, with post-hoc).

Due to the accentuated stabilization of the curves, the best model that fits these results is a cubic association. In the 1 hour exposure, this model suggests a dose-response relationship with a coefficient (R^2) of 0.806, and a curve equation of $y = 0.006x^3 – 0.437x^2 + 8.909x + 23.974$; on the other case, with an exposure of 3 hours, the best fitting curve has an R^2 of 0.858 and an equation of $y = 0.654x^3 – 10.557x^2 + 45.722x + 14.819$. However, since a cubic model does not have a logical biological meaning, these regression results were not considered to be reliable and, consequently, were not represented in Figure 19.

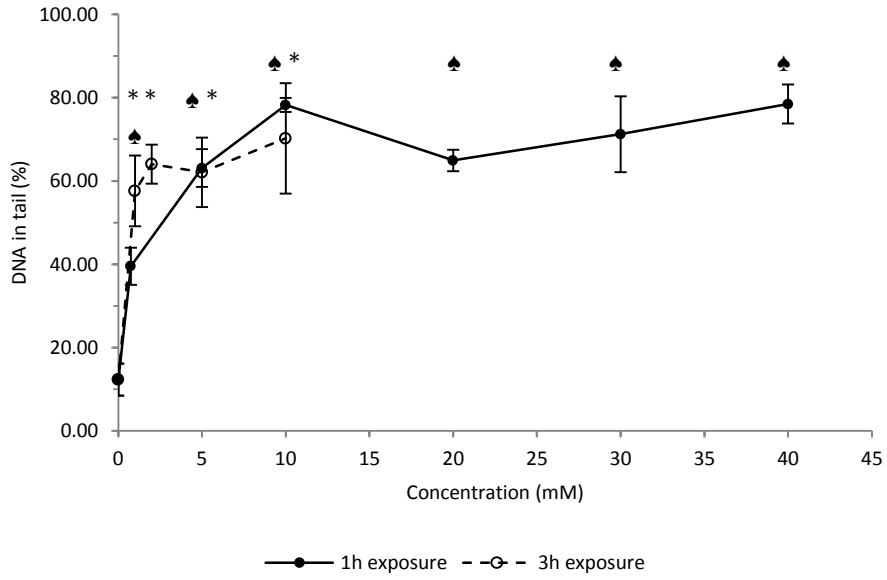


Figure 19. Results of modified Comet Assay with EMS on A549 cells. Significantly different from the control (p<0.01, One-Way ANOVA test): ♠ – 1h exposure; * – 3h exposure.

As before, the resulting curve of the oxidative DNA damage has a unique behavior, increasing at first, and decreasing after a certain value, observable in Figure 20. This is due to the stabilization of the curve of the percentage of DNA damage in the FPG-treated cultures. In the 1 hour exposure, the first three concentration of EMS (0.75, 5 and 10 mM) are significantly different from the control (p=0.04 in the first case and p<0.001 in the others); in the 3 hour exposure, the concentrations of 1 and 2 mM also have statistical significance (p=0.004 and p=0.03). Regression analysis, however, does not offer any model that fits these curves, and therefore, no dose-response association can be made in terms of oxidative damage.

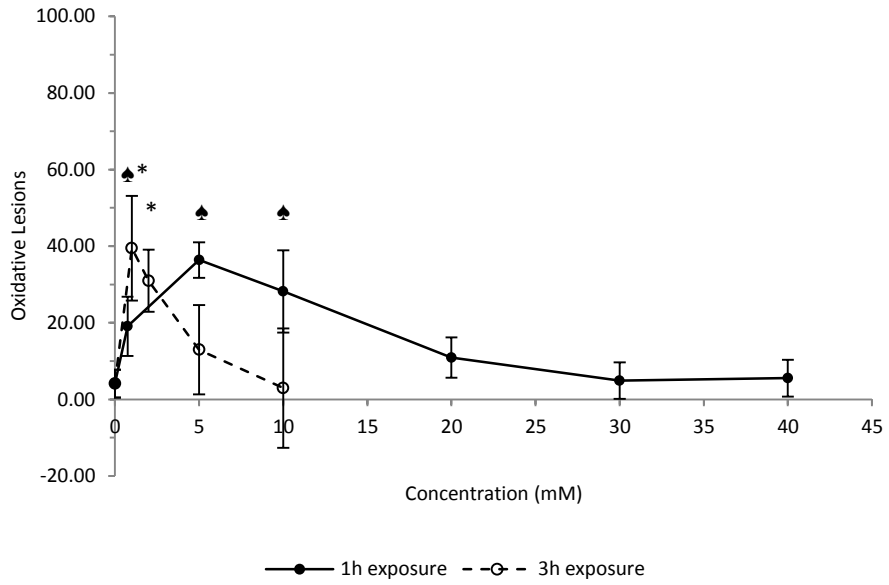


Figure 20. Oxidative damage caused by EMS on A549 cells. Significantly different from the control (p<0.05, One-Way ANOVA test): ♠ – 1h exposure; *- 3h exposure.

The comparison of the oxidative lesions results obtained in the two cell lines is presented in Figure 21.

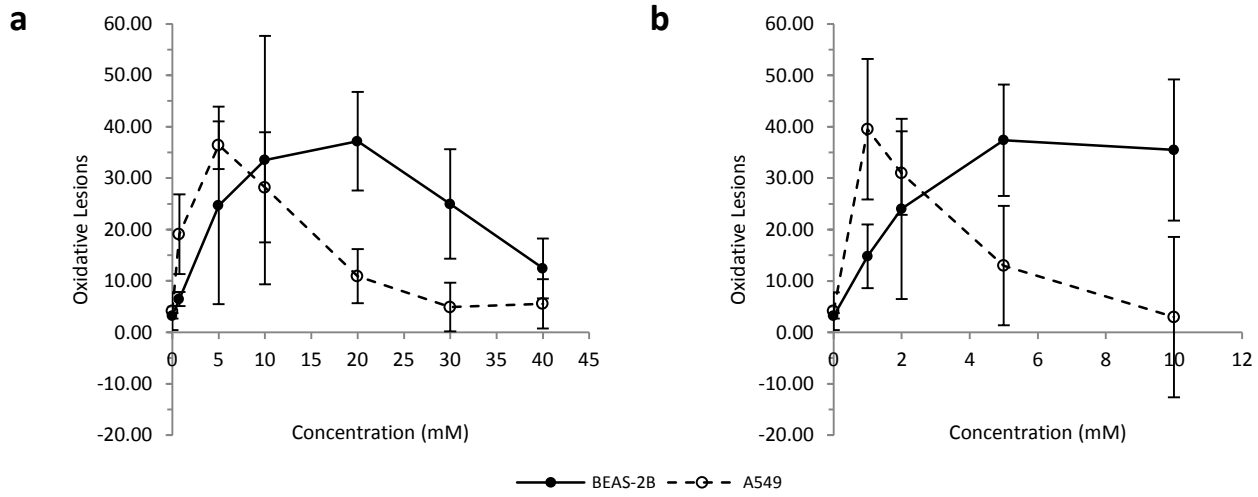


Figure 21. Comparison of the results of oxidative damage caused by EMS on BEAS-2B and A549 cell lines. **a** – 1 hour exposure; **b** – three hours exposure.

3. Cytotoxicity of the MWCNT

a. Cell counting

In the case of the BEAS-2B cells, after the cell treatment with the nanomaterial, the results of the cell counting (Figure 22-**a** and 22-**c**) showed that none of the tested MWCNT was significantly cytotoxic on BEAS-2B cells after a short exposure, since the cell cultures never reached a 50% decrease of viability (p>0.05, Student's t-test). However, due to the fact that this is a very short exposure period, the viability of

the cells would only be significantly altered if the nanomaterials caused a strong acute toxicity. In the case of the long term exposure – 24 hours – some nanomaterials displayed a significant decrease of viability at some of the doses: NM-400 at the highest dose (p<0.01), and NM-401 at the doses of 16, 64 and 128 μg/cm^2 (p=0.03, p<0.01 and p=0.03, respectively). Furthermore, the results for NM-401 with the 24 hour exposure time showed a dose-response relationship, which fits to a logarithmic curve (curve equation: y = -2.165 ln(x) + 75.93; R^2=0.9133).

Regarding A549 cells exposed to NM-401, the result is consistent with the observations in BEAS-2B, since the viability is mostly unchanged with the variation of nanomaterial concentration (Figure 22-**b** and 22-**d**). In the short exposure time, and possibly due to the large standard deviations of the mean value, none of the results showed statistical significance (p>0.05). In the case of the long term exposure, the concentration of 64 μg/cm^2 is the only one that produced a significant and consistent decrease of viability comparatively to the negative control (p<0.01).

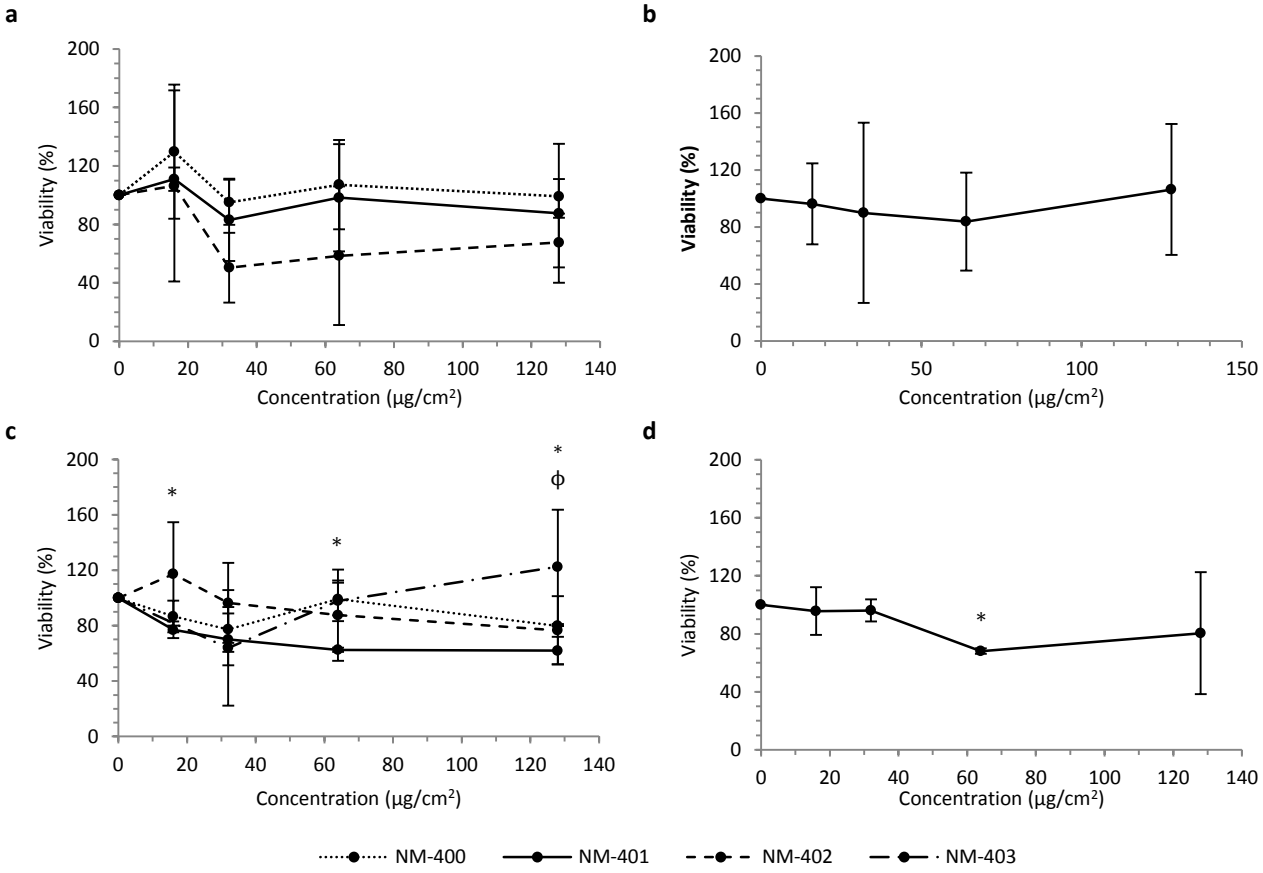


Figure 22. Results of the cell counting on BEAS-2B and A549 cells exposed to MWCNT. **a** and **c** – BEAS-2B after 3 and 24 hours, respectively; **b** and **d** – A549 after 3 and 24 hours, respectively. Significantly different from the control (p≤0.05, Student's *t*-test): * – NM-401 and ϕ – NM-400. The results of the positive control (EMS) can be found in the Annexes.

b. Clonogenic Assay

The results of the Clonogenic assay in A549 cells exposed to the nanomaterials can be seen in Figures 23 and 24. The first one represents the aspect of the plate wells with the cell colonies in the negative control (Figure 23-a) and treated with 128 μg/cm^2 of NM-401 (Figure 23-b). It is evident that the number of colonies is considerably reduced in treated cells. The number of cells within each colony is also substantially smaller in that plate well, noticeable by the reduction of the size of the colonies.

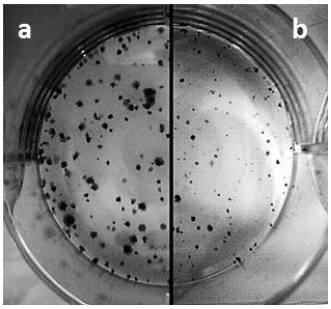


Figure 23. Clonogenic assay results: negative control (**a**) vs. following NM-401 treatment in a concentration of 128 μg/cm^2 (**b**).

In Figure 24, it is possible to note that all three nanomaterials caused a significant decrease in the proliferative capacity of the cells – in all concentrations of NM-401 (p<0.01 in all doses; t-test), and in the last three of NM-402 (p=0.05, p<0.01 and p<0.01, respectively) and NM-403 (p=0.02, p=0.03 and p<0.01, respectively).

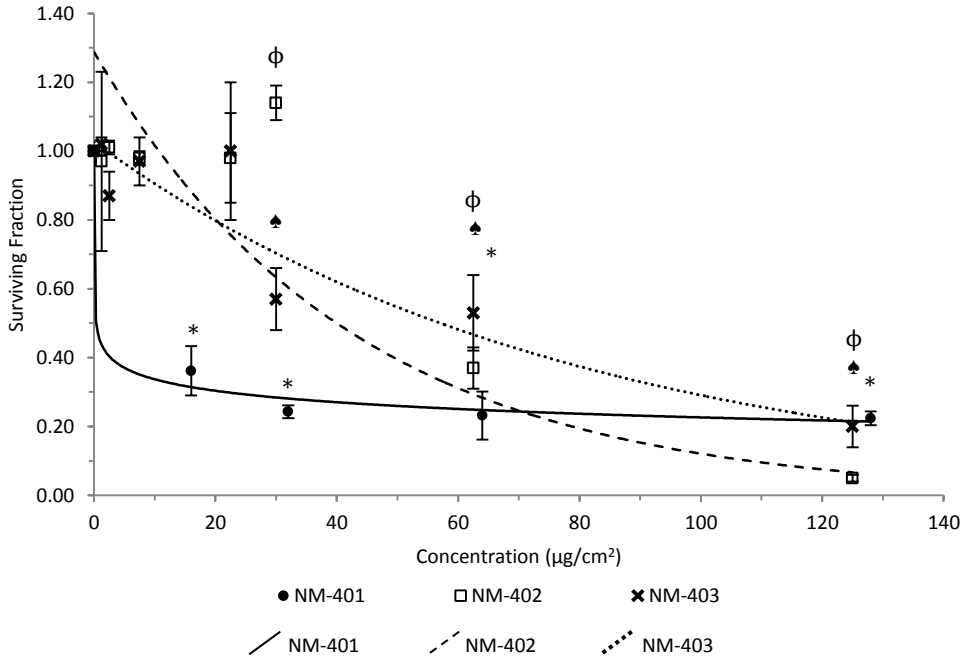


Figure 24. Results of the clonogenic assay, on A549 cells, after 8-10 days exposure to each nanomaterial. Significantly different from the control (p≤0.05, Student's t-test): * - NM-401; ϕ - NM-402 and ♠ - NM-403. The results of the positive control (MMC) can be found in the Annexes.

All three curves were subjected to a regression analysis, and the best curve that fitted the data was drawn in each of them. A summary of the results may be found in Table 5.

Table 5. Conclusions of the regression analysis performed on the results of the clonogenic assay on A549 cells, after 8-10 days exposure to each nanomaterial.

Nanomaterial	Model	Equation	R^2	IC50 (μg/cm^2)
NM-401	Logarithmic	$y = -0.048 \ln(x) + 0.448$	0.9910	-
NM-402	Exponential	$y = 1.2878\, e^{-0.024x}$	0.9103	39.42
NM-403	Exponential	$y = 1.027\, e^{-0.013x}$	0.9329	55.37

Due to the lack of information concerning the effects of small doses of NM-401 (0-16 μg/cm^2), the equation suggested by the regression analysis for this nanomaterial is not considered biologically relevant and, for that reason, the value of IC50 of this MWCNT was not calculated.

c. Cytokinesis-blocked proliferation and replication indexes

The micronucleus assay also provides information concerning cytotoxicity, in the form of proliferation and replication indexes.

In BEAS-2B cell line, NM-400 caused a considerable change in both indexes: all the values were significantly different from the controls, with steady positive slopes. The CBPI results were significantly different from the negative control with *p*-values of 0.04 in the lowest concentration, and 0.02 in all the others (*t*-test); the RI results exhibit *p*-values of 0.04 in the first two doses, and 0.03 and 0.05 in the last two, respectively. NM-402 caused a significant increase in the proliferation index as well, but only in the concentrations of 64 and 128 μg/cm^2 (p=0.014 and 0.023, respectively; One-Way ANOVA, with post-hoc). These results are represented in Figure 25.

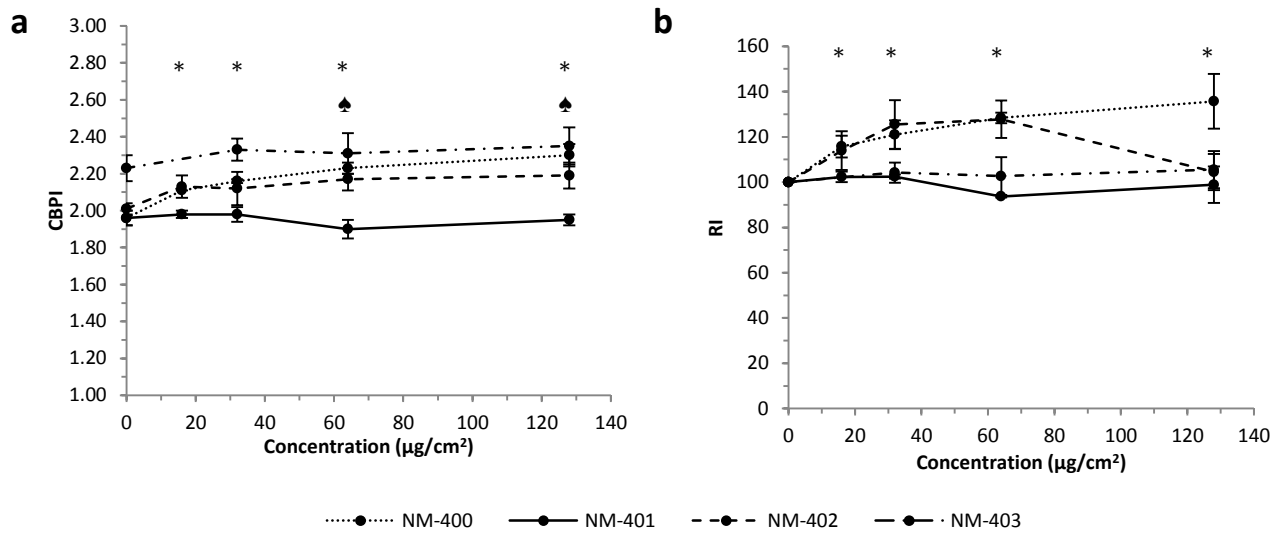


Figure 25. Results of the CBPI and RI of BEAS-2B cells exposed to MWCNTs: **a** – CBPI; **b** – RI. Significantly different from the control: * – NM-400 (p<0.05, Student's t-test), ♠ - NM-402 (p<0.05, One-Way ANOVA). The results of the positive control (MMC) can be found in the Annexes.

In the case of the A549 cells, NM-401, unlike any of the other nanomaterials, also caused a significant change in cell cycle: both the proliferation and the replication indexes are significantly different to those of the control. CBPI decreases steadily, having a significant result in the highest concentration (p=0.025, t-test); RI has the same behavior, consistently reducing, but with significant differences only in the concentrations of 32 and 128 µg/cm^2 (p=0.033 and 0.004, respectively; t-test). These results are represented in Figure 26.

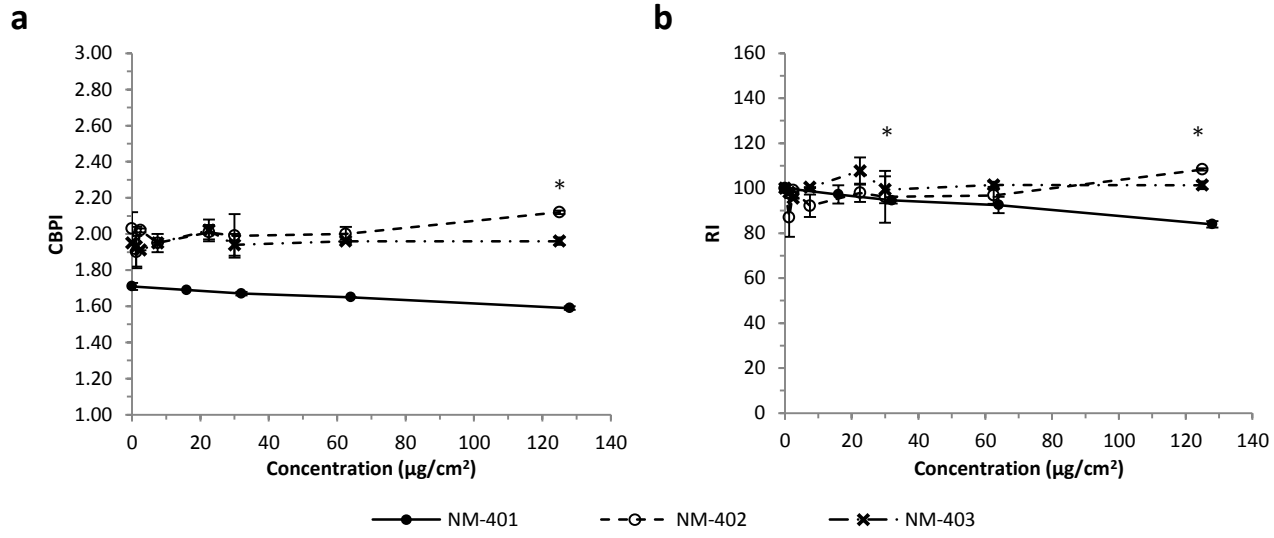


Figure 26. Results of the CBPI and RI of A549 cells exposed to MWCNTs: **a** – CBPI; **b** – RI. Significantly different from the control (p<0.05, Student's t-test): * - NM-401. The results of the positive control (MMC) can be found in the Annexes.

In summary, the cytotoxicity results concerning the nanomaterials in BEAS-2B and A549 cells are mainly negative, and sometimes conflicting between assays. Interestingly, in A549 cells, the clonogenic assay has identified all the tested carbon nanotubes as toxic, unlike the cell counting or the indexes.

4. Genotoxicity of the MWCNT

a. Comet Assay

Concerning the exposure of BEAS-2B cells to nanomaterials, the results obtained in the unmodified Comet Assay (Figure 27) show that none of the nanomaterials caused a significant alteration on the percentage of DNA in tail, regardless of the exposure time, with the exception of NM-403, after an exposure of 24 hours to the concentration of 32 and 128 μg/cm^2 ($p \leq 0.05$, One-Way ANOVA test). However, since the difference between these results and the negative control is reduced, it will not be considered as having a relevant biological meaning.

The assay was also modified by the addition of FPG enzyme. It is clear that, similarly to the unmodified assay, none of the nanomaterials caused a significant change on the % of DNA in the comet tail, in either of the exposure times.

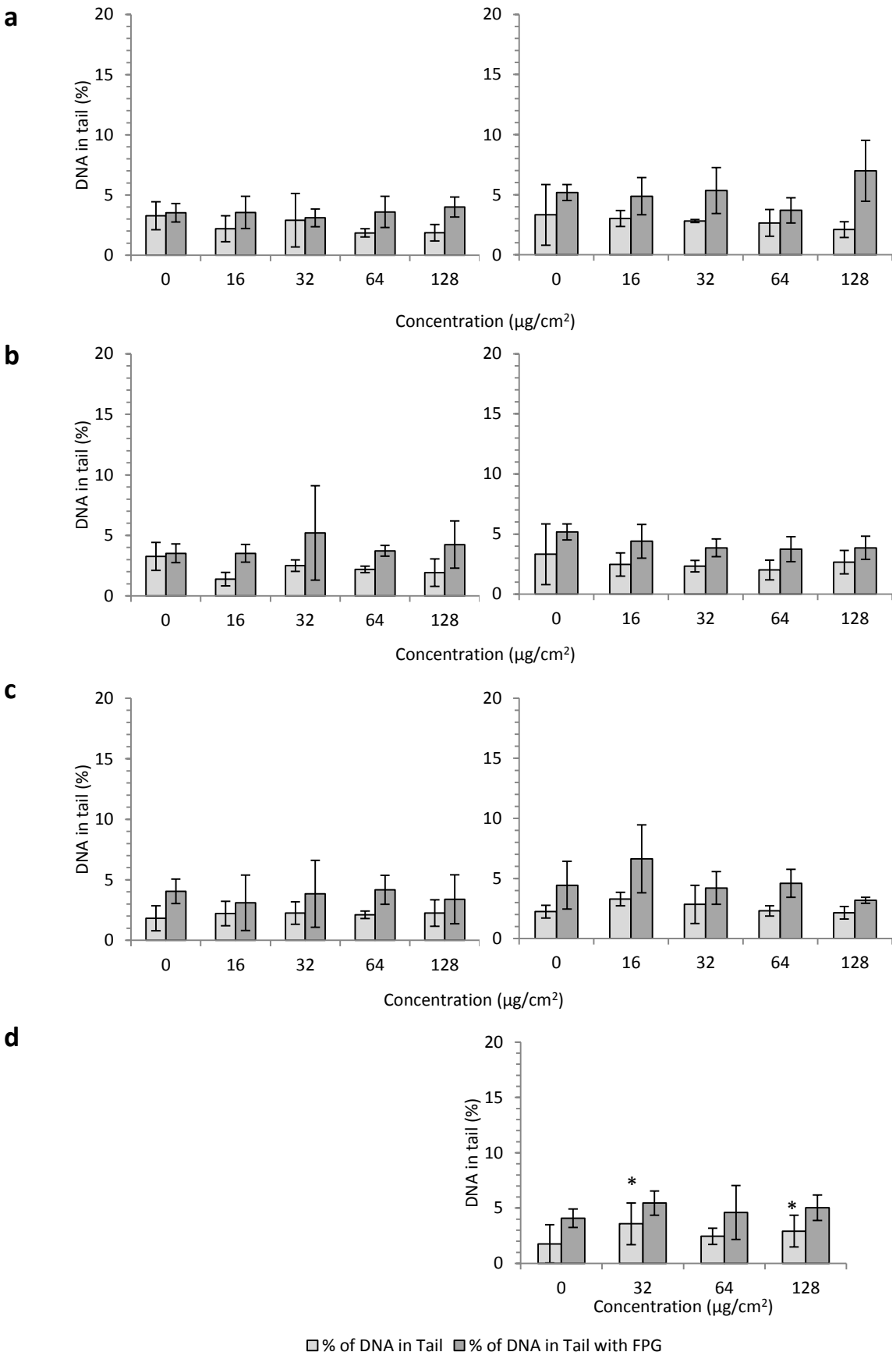


Figure 27. Results of the Comet Assay in BEAS-2B cells exposed to MWCNT; left – 3 hours exposure, right – 24 hours exposure. **a** – NM400; **b** – NM-401; **c** – NM-402; **d** – NM-403. Significantly different from the control ($p \leq 0.05$, One-Way ANOVA test): * – NM-403. The results of the positive control (EMS or H_2O_2) can be found in the Annexes.

Regarding the A549 exposure to the nanomaterials, the results obtained either in the unmodified Comet Assay or in the FPG-modified version (Figure 28) show that none of the nanomaterials caused a significant alteration in the level of DNA damage, regardless of the exposure time.

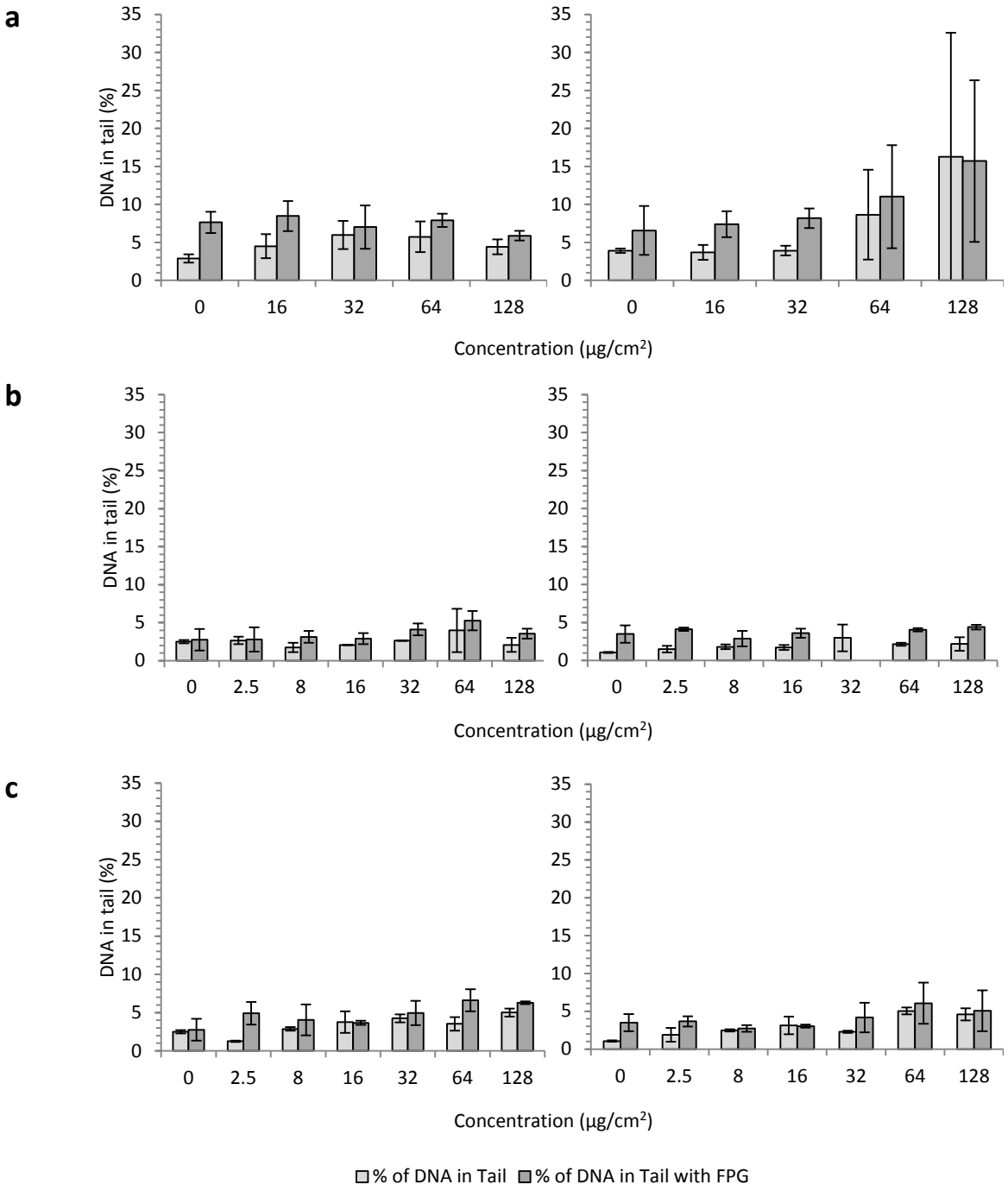


Figure 28. Results of the Comet Assay in A549 cells exposed to MWCNT; left – 3 hours exposure, right – 24 hours exposure. a – NM-401; b – NM-402; c – NM-403. The results of the positive control (EMS or H_2O_2) can be found in the Annexes.

b. Micronucleus Assay

With the exception of NM-403, none of the carbon nanotubes caused any significant instability to the chromosomes of the BEAS-2B cells – seen by the lack of a change in the frequency of micronuclei in binucleated cells (MNBNC). Only NM-403, in the concentration of 32 μg/cm^2 produced a significant difference in this number (p=0.015; Fisher's Exact Test), showing a decrease in MNBNC.

In Figure 29, it is possible to observe the results of the Micronucleus assay in BEAS-2B cell line, regarding the number of micronucleated binucleated cells per 1000 binucleated cells.

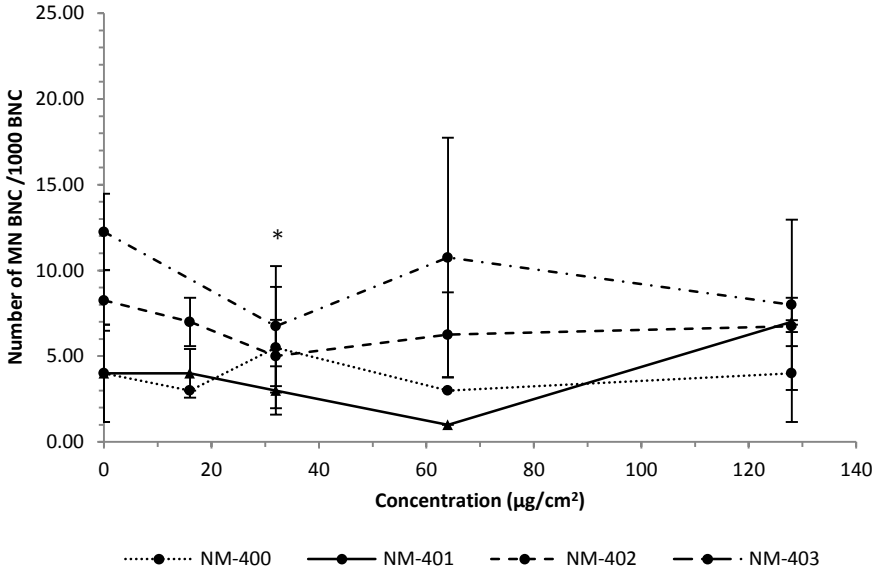


Figure 29. Results of the Micronucleus Assay in BEAS-2B cells exposed to MWCNTs: MN BNC/1000 BNC. Significantly different from the control (p<0.05, Fisher's Exact test): * – NM-403. The results of the positive control (MMC) can be found in the Annexes.

In terms of changes in the frequency of micronucleated binucleated cells in A549 cells (Figure 30), all the tested carbon nanotubes have produced statistically significant differences comparatively to the controls. NM-401 and NM-402, on one hand, caused a significant increase in the frequency of micronucleated binucleated cells, in comparison to the respective controls: the highest concentration of NM-401 significantly increased this frequency, with a p=0.001 and the two highest doses of NM-402 approximately doubled it (p=0.006 and 0.019, respectively; Fisher's Test). NM-403, on the other hand, caused a decrease in the frequency of micronucleated cells to approximately half of that of the control, in the concentrations of 7.5 and 22.5 μg/cm^2 (p=≤0.05).

Regression analysis indicated that, regarding NM-401 and NM-402, there is a significant dose-response relationship which fits, in both cases, a quadratic model: NM-401 has a curve equation of $y=0.001x^2 -0.091x + 5.877$, and a correlation coefficient of R^2=0.981, and NM-402 has a curve equation of $y=-0.0004x^2 + 0.1638x + 6.9895$, and an R^2 of 0.861.

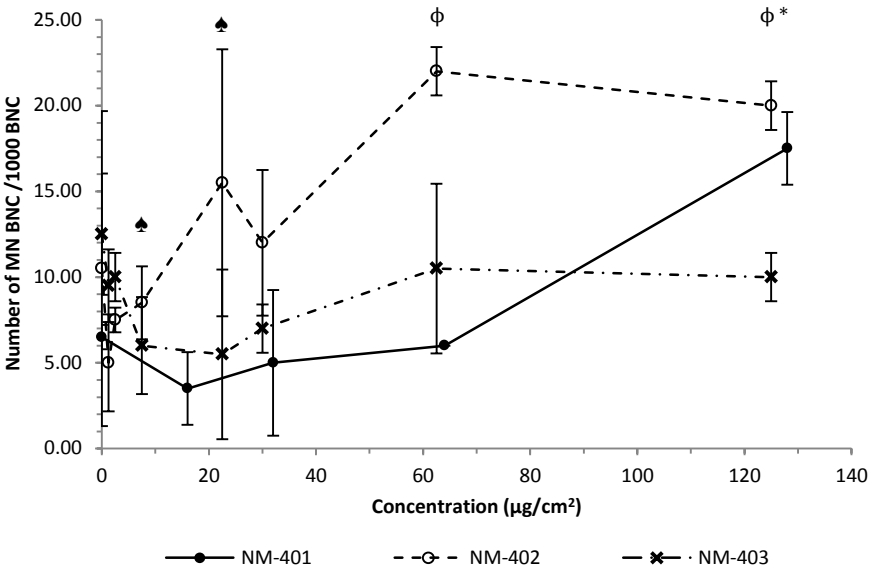


Figure 30. Results of the Micronucleus Assay in A549 cells exposed to MWCNTs: MN BNC/1000 BNC. Significantly different from the control (p<0.05, Fisher's Exact test): * – NM-401, ϕ – NM-402 and ♠ – NM-403. The results of the positive control (MMC) can be found in the Annexes.

Discussion

Nanotechnology is becoming more and more essential in industry and science and it is believed that its importance will grow even further in the future. The use of nanomaterials is justifiable due to the broad range of applications in almost every aspect of life, such as in electronic devices, transportation, mechanical industry, clothing, healthcare products, medical procedures and many more. However, the widespread use of nanomaterials can also have unexpected consequences to human health and to the environment, as their valuable characteristics can also influence short and long term negative effects, such as tissue inflammation, asthma, ulcers or even cancer [35]. The lack of scientific knowledge concerning these aspects makes the establishment of relevant legislation a challenge; this project intends to support these decisions, since it aims to provide some evidence about the safety, or risks, related to the use of carbon nanotubes. In order to reach this goal, both cytotoxicity and genotoxicity were tested for a series of multi-walled carbon nanotubes, in two human respiratory tract cell lines. The results were then compared to the nanoparticles' characteristics, in an attempt to uncover if there is a specific physical property that contributes most to the cytotoxic or genotoxic potential of these CNT.

Even though there is a large amount of scientific research in this area, most of the published results are contradictory and/or confusing, and the majority of the studies have methodological differences that do not allow direct comparison of results. The preparation of the nanomaterial solutions, including the media and the protocol used for the particle dispersion, differ greatly between studies, as well as the general procedures used in the cell culture exposure and in the toxicity assays. Different nanomaterials have, evidently, different characteristics, and even closely related nanomaterials (as the carbon nanotubes studied in this work) need to be thoroughly characterized in terms of physical and chemical properties.

Throughout the various sections within this chapter, in order to better visualize the outcome of the project, the results will be summarized in a series of tables (7 and 8). The meaning of the symbols used in them is clarified below, in Table 6.

Table 6. Description of the symbols used in the summary tables presented in this chapter.

Symbol	Meaning
+	**Positive Results**: the results showed a statistically significant alteration in two or more concentrations in comparison to the control or, alternatively, a statistically significant change in the highest concentration combined with a dose-response relationship.
(+)	**Equivocal Results**: the results showed a statistically significant change in only one concentration; it was not possible to establish a dose-response association.
-	**Negative Results**: the results did not show any significant difference when compared to the control sample.
NP	**Not Performed**

1. Qualitative Analysis of the Dispersion of the tested Nanomaterials

As was previously described, the protocol for the dispersion of the nanomaterials is a generic procedure developed within NANOGENOTOX Joint Action, as the best approach to disperse all the nanomaterials included in that project (titanium dioxide and synthetic amorphous silica, as well as the carbon nanotubes) [56]. In fact, this protocol is in agreement with other authors who have demonstrated that a small concentration of protein improves the dispersion of nanomaterials [72]; however, there is also the possibility that this addition of protein to the dispersion medium may influence the nanoparticles' surface properties and their interaction with other molecules [52], as well as their toxicity [73].

This protocol is generally considered appropriate for the dispersion of the majority of nanomaterials, and Tavares and colleagues, who tested the same nanomaterials that were used in this work, confirmed, using dynamic light scattering (DLS) and transmission electron microscopy (TEM), that it produced stable dispersions. However, the authors also demonstrated the existence of a large number of aggregates/agglomerates, and an increase in the size of these masses with nanomaterial concentration, which might interfere with the results [32]. The use of DLS in this case is considered not to be entirely appropriate, though, due to the fiber-like morphology of the carbon nanotubes, which is problematic for the interpretation of the results; in this case, the authors also reported that the results of this analysis may contain not only signals from the carbon particles, but also from impurities present in the solutions [32].

Regardless of these reports concerning the quality of this procedure, during the course of this work, some problems concerning the maintenance of the homogeneity of dispersions could be observed. For all nanomaterials studied, the difference between the stock dispersion before and after the dispersion protocol was quite evident in terms of number of individualized particles, which suggests the dispersion was, indeed, successful. Nevertheless, the dispersion did not appear to stay homogeneous over a period of time, and a large part of the particles adhered to the vial glass walls where the dispersions were prepared. This phenomenon may have impact on the effective concentration of the dispersions to which the cells were exposed. However, this is considered to be a problem related more to the class of nanomaterials in question than to the dispersion protocol used; for this reason, this is probably an unavoidable occurrence in every study in which these carbon nanotubes are analyzed in liquid medium – the interference with the analysis of the results has been documented by several authors, who used different dispersion protocols, such as Tavares *et al.*, Corradi *et al.*, or Lindberg *et al.* [32, 74, 75]. Furthermore, the comparison of the appearance of the four nanomaterials in BSA-water dispersion proved that the different characteristics of the particles caused changes in the quality of the dispersions obtained, namely its homogeneity. The figures presented in the **Results** chapter document the general macroscopic aspect of the MWCNT suspensions at different time points, showing that the homogeneity of the suspensions is gradually lost over the exposure time. These photographs suggest that, of the three MWCNT presented in the images, NM-401 presents the most homogeneous dispersion over a period of time, while NM-400 is the CNT that loses dispersion quality

faster – these evaluations are based on the size of the visible agglomerates and aggregates in the plate wells and in the glass vials and thus represent a qualitative appreciation only. Furthermore, it is necessary to take into account the tendency of the nanoparticles – particularly hydrophobic ones, like carbon nanotubes – to sediment. In Figure 12, it is clearly observable that these nanomaterials experience great sedimentation over time, accumulating on the bottom of the plate wells. This deposition may happen without necessarily occurring agglomeration of the particles. Therefore, the evaluation of the homogeneity of the particles dispersion presented above (NM-401 > NM-402 > NM-400) based on its visual aspect over time is possibly inaccurate, as the contribution of agglomeration/aggregation and sedimentation to the overall aspect of the suspensions is not taken into account.

In the work by Tavares *et al.*, a characterization of the nanomaterial dispersion and sedimentation in culture media was performed with DLS and TEM. The authors documented a slow sedimentation in suspensions of NM-400 and NM-402, as well as a limited deposition of NM-403 particles; in the case of NM-401, 25% of the particles had deposited after 6 hours [32]. Concerning the state of the dispersions of the nanomaterials, the authors' results pointed to a completely opposite classification to the one made above: NM-400 appeared to produce the finest dispersion, while NM-401 produced the coarsest one [32]. It is possible that these measurements support the argument mentioned above: the sedimentation of the particles may not be associated to agglomeration or aggregation of the particles, but simply accumulation of the coarsest particles at the bottom of the vials [32]. It is important to note, though, that this study was performed on a culture medium different from those used in the present work, which may help explaining the different sedimentation pattern.

Because the characterization of the suspensions in the present work was based solely on optic observation, which is prone to error and misinterpretation, the evaluation made by Tavares *et al.* [32] will be preferentially used to further discuss the results of the genotoxicity assays. The four nanomaterials were assigned a number 1-4 considering the characterization of the dispersion (1 being the best dispersed, 4 being the worst). It was also attempted to associate the state of the dispersion to the characteristics of the particles, such as length, diameter, surface area and aspect ratio, but no clear association could be obtained. It was concluded that the homogeneity of the dispersion was unaffected by the mentioned physical or chemical properties of the carbon nanotubes; possibly other properties could be associated to this parameter, but no information was provided by the manufacturers.

The agglomeration and aggregation of the nanotubes may be problematic in the *in vitro* exposures, due to the fact that the cells are exposed to an amount of dispersed nanomaterials as well as to large agglomerates or carbon nanotubes. Most likely, the cell culture was being subjected not only to possible cyto- and genotoxic effects by the individualized particles, but also to physical aggression caused by the addition of vast masses of particles to the growth medium, producing damage unrelated to the

nanomaterials' characteristics. Besides from these aspects, the deposition of nanoparticles also interferes in the observation of the results.

However, this is likely a natural part of the nanomaterial behavior outside the controlled laboratory conditions and, therefore, the complete dispersion of the nanomaterial aggregates or agglomerates may not be relevant for human health [56]. Especially in the lungs, where the particles come in direct contact with the lung lining fluid immediately upon exposure and then with the epithelial cells, it is highly likely that they reagglomerate/reaggregate even before reaching the cells [52]. It is thought that agglomerated nanotubes, being heavier, deposit higher in the airways, while dispersed particles, being lighter and more easily carried along with the breathed air, deposit in deeper levels of the airways, such as the alveoli and the interstitial space; this suggests that different areas of the airways are exposed to different concentrations of CNT. Besides, outside of the lungs, in the blood stream or in other organs, nanoparticles will interact with proteins and other molecules, which will change their behavior over time [52].

In addition, this method could not mimic a possible *in vivo* or real-life inhalatory exposure scenario in this case, as this route of exposure requires airborne particles, not nanomaterials dispersed in a liquid medium. In works studying the toxicity of CNT with this exposure route, the dispersion in a liquid has disadvantages, such as the occurrence of changes in the surface of the particles or the attachment of ions or proteins [56].

Due to the fact that scanning electron microscopy only allows for the observation of the external features of the objects, the images presented in Figure 13 do not clarify if the nanoparticles actually penetrated the membranes of the cells and reached the cytoplasm and organelles, or if they are just attached to the exterior of the cells – to have this confirmation, it would be necessary to use Transmission Electron Microscopy to check the interior of the tissues. If the fibers actually enter the cells, they may interact with the cellular organelles, disrupt membranes, interfere in biochemical mechanisms, or even destroy or replace cellular elements (as described in the **Introduction**, and exemplified in Figure 2). If this is the case, it is expected that these nanomaterials have quite clear cytotoxic and/or genotoxic effects. However, if it is not – if the carbon nanotubes are not able to pierce the membranes of the cells or enter through endocytosis – the lack of positive results in viability and genotoxicity assays is quite plausible. This aspect has been investigated by several authors, whose results will be discussed ahead, in the **Viability** section.

2. Tests for the Validation of the Methods – Ethyl Methanesulfonate

Throughout this study, it was determined that the concentration of EMS that was being used as a positive control in the shortest exposure period (0.75 mM for three hours) was not high enough, as the

results were similar to those of the negative control (see **Annexes**). For comparison of the results with those of NANOGENOTOX, this concentration was still used in all the assays of this work; afterwards, other concentrations of EMS were tested, so that the most suitable one could be chosen as a positive control in future experiments.

Initially, in order to analyze the influence EMS has on the viability of human cell lines, the cell counting method was performed after two exposure times: one and three hours. The results showed that this chemical does not have a strong cytotoxic effect in either of the cell lines, and in either of the exposure periods. The graphs exhibit fluctuating lines, indicating increases in viability immediately followed by decreases, which shows the general variability of this assay. The results are in harmony with those obtained by Doak *et al.*, who tested the cytotoxic and genotoxic potential of EMS in a human lymphoblastoid cell line (AHH-1); in terms of reduction of viability, the results were mainly negative despite some fluctuations in the data [76]. However, the authors used a range on concentrations of 0-2.5 µg/mL (0-0.02 mM) which is extremely low in comparison to the concentration range used in the present work. Kimura and colleagues, on the other hand, obtained different results: the authors exposed another human lymphoblastoid cell line (TK6) to EMS (62.5-2000 µg/mL = 0.5-16 mM), tested the viability of the cells after three exposure times, and concluded that the cell viability decreased proportionally to the exposure time [77].

Afterwards, the genotoxicity of this compound was tested with the Comet assay, with the same two exposure times. In both exposure times it produced an increase in the percentage of DNA in the comet tails. In both cell lines, several values were significantly different from the control, which shows that EMS is, as expected, highly genotoxic [78, 79]. Comparing the results from the EMS exposure of the two cell lines, it is also possible to conclude that A549 cells are apparently more susceptible to this chemical than BEAS-2B cells. When FPG was added to the assays, it was possible to observe that, in both cell lines, the percentage of damage was higher than in conventional comet assay and stabilized at the higher doses. In BEAS-2B cells, this steadiness was more evident after only one hour than it was after three; this may be due to the smaller doses used in the second case, or to a situation where the DNA damage was already being repaired after some time, reaching a steady state. In A549 cells, this stabilization it quite obvious at both exposure times, even decreasing slightly in one of the doses in the first case.

The comparison of the data from the conventional and the modified comet assays results in the amount of oxidative lesions caused by this substance. It was observed by this comparison that EMS induces oxidative DNA lesions. In both cell lines, the percentage of oxidative damage increases steadily at first, but at higher concentrations, it either stabilizes (BEAS-2B, 3h exposure) or markedly decreases (A549, both exposure periods). Comparing the two cell lines, it also becomes evident that in lower doses, EMS causes more oxidative lesions in A549 cells, and in higher doses, in BEAS-2B cells (Figure 21). This suggests that there are differences in the uptake of this substance by the cells, or different susceptibilities by the two cell lines to EMS.

The genotoxic potential of EMS has been described often in the literature, and this chemical has been regularly used as a positive control due to its highly clastogenic and mutagenic effects [79]. Sega has written an extensive review of some works published at the time which used EMS, either as the subject of the test, or as a positive control [78]. In this work, the author reveals that the mutagenicity of EMS may be partially explained by its alkylating action on the purines and/or pyrimidines present in the DNA chain, making them highly unstable [78, 79]. This process may possibly lead to breaks, or to an attempt by the cell to repair the damaged area by base excision repair [79]. This is performed by removing the affected base and, consequently, generating apurinic and apyrimidinic sites. When DNA synthesis occurs, a randomly selected base will be inserted to pair with the abasic site, producing mutations [78]. Another possible cause is the alkylation of the phosphates present in the nucleic acids, which could inactivate the molecules or lead to their hydrolysis on the altered site, generating breaks [78].

Doak and colleagues, as mentioned above, tested EMS for its ability to cause DNA damage, and were able to observe a significant increase in the frequency of micronuclei, as well as in the amount of induced mutations, on a human lymphoblastoid cell line (AHH-1) exposed to EMS in a concentration of 1.4 µg/mL (0.01 mM); although, there was not a linear dose-response [76]. Kimura *et al.* tested EMS (62.5-2000 µg/mL = 0.5-16 mM) on a related cell line (human lymphoblastoid, TK6) and discovered a significant increase in both % of DNA in the comet tails and number of micronucleated binucleated cells, associated to the exposure dose [77]. Wagner and co-workers studied the influence of EMS (0.5-15 mM) in the genome of Chinese Hamster Ovary cells with Comet and Micronucleus assays, and verified that the level of DNA damage significantly increased in both assays, and had clear dose-response associations; besides, the results of the two assays had a high correlation [80].

In summary, the results of this project suggest that EMS is, in fact, highly genotoxic in human lung and bronchial cells, but not cytotoxic under the analyzed conditions. In the next section, a critical analysis will be made of the cytotoxicity assays performed in this project, especially the cell counting step. As will be explained, this is not a very reliable method for the assessment of viability, and more tests should be employed to more accurately measure the cytotoxicity of this compound.

As was mentioned in the beginning of this section, the optimization of the methodologies by testing several concentrations of EMS had the main goal of choosing an adequate dose to be used as a positive control in future studies. According to the OECD, the purpose of a positive control is to demonstrate the sensitivity of the test protocol and the cells and, for this reason, a substance used as a positive control should cause a small but clear positive response in the tested system. Besides, the viability of a cell culture exposed to a positive control should not be lower than 50% [81].

In this work, the analysis of the cytotoxicity results of the cells exposed to EMS revealed that the viability of both cell lines is always higher than 50% in this concentration range, suggesting that this parameter should not influence the choice of the optimal concentration of EMS as a positive control.

Regarding genotoxicity, it is necessary to consider each cell line separately, due to possibly different susceptibilities of the cells to this chemical. In the case of BEAS-2B cells, the concentration of 10 mM with an exposure of 1 hour appears to be appropriate, causing a level of DNA damage of approximately 17.5%; with the addition of FPG, this value increases to 47.5%. In the longer exposure period (3 hours), the concentration that best seems to fit the above criteria is 5 mM, even though the level of DNA damage is higher than in the previous situation: 20.2% of DNA in tail, 58.5% with FPG. In these two situations, the amount of oxidative lesions is similar, in the range of 29.5-38.6%.

The A549 cell line seems to be much more sensitive to this substance than BEAS-2B, since the same range of DNA damage is obtained after an exposure to much lower doses. In the shorter exposure time, the concentration of 0.75 mM caused approximately 21.3% DNA in comet tail, and 30.4% with the addition of FPG. This EMS concentration was the same that was used in the nanomaterial assays and, as stated above, did not produce adequate results after an exposure of 3 hours (3-7% DNA in tail, similar to the negative control). This difference between the results of a longer and a shorter exposure periods to the same concentration may be explained by a possible damage repair mechanism: after 1 hour, EMS causes 21.3% damage in the DNA of A549 cells, which is partially repaired in the following 2 hours, leading to small comet tails and, consequently, an apparently inadequate positive control.

In the longer exposure period, this range of damage is obtained after exposure to 1 mM (20.4% DNA damage without FPG, 50.6% with FPG). The range of results of oxidative lesions caused by EMS in this cell line and in these concentrations is much wider than in the previous case: 9.1-30.2%.

3. Viability

Measuring viability in *in vitro* cell models exposed to nanomaterials exhibit many challenges, such as the agglomeration/aggregation of the particles in the exposure medium, the binding of the nanoparticles to the cells, the interference of the materials in the visual observation of the results and the complexity in the analysis of the data due to the large amount of variable parameters (nanoparticle length, diameter, surface area, etc.). The analysis of the results needs be performed critically, with a solid knowledge of how the method works and how the particles may behave in that particular situation. The interpretation of the data must be logical and should not be based on one assay only: at least two different cytotoxicity assays should be performed in each case, in order to compare the results and the endpoints being analyzed [52]. It is also

important to note that the carbon nanotubes used in the literature often have different characteristics than those studied in this project, which prevents a linear comparison between the results.

Several authors recommend the use of more than one cytotoxicity assay, due to the fact that some nanomaterials interact with dyes or products of the metabolism of the dyes by the cells, or interfere with the observation of the results (as in the case of spectrophotometer-based assays, such as Neutral Red) [82].

In this project, three endpoints were analyzed to determine cytotoxicity: cell counting coupled to the trypan blue exclusion assay, the clonogenic assay and determination of the replication index. Cell counting relates the number of viable cells counted following cells exposure to the nanomaterials with the number of non-exposed cells at the same time point. The clonogenic assay evaluates the potential of the cells to replicate and, therefore, form colonies after the exposure to the test agent [62]. The replication index (RI) measured in micronucleus assay is calculated to evaluate whether the cells have undergone mitosis in the course of the assay, and to determine how many cell cycles the cells have completed, in comparison to the control cells [81].

The viability results are summarized in Table 7; the NM-403 results, as was mentioned above, were previously obtained by this research group using the same procedures, and are included here for global comparisons.

Table 7. Summary of the cytotoxicity results.

Cell Line		**BEAS-2B**			**A549**			
Assay		Cell counting/ Trypan Blue		CBPI or RI	Cell counting/ Trypan Blue		Clonogenic	CBPI or RI
Exposure		3 hours	24 hours		3 hours	24 hours	8 days	
Nanomaterial	NM-400	-	(+)	+	NP	NP	NP	NP
	NM-401	-	+	(+)	-	(+)	+	+
	NM-402	-	-	(+)	NP	NP	+	-
	NM-403	NP	-	-	NP	NP	+	-

The comparison of the present results with those reported in other studies is not direct, due to the fact that the nanomaterials used in each study have different characteristics (sometimes not described). Guo *et al.*, for example, used the Trypan Blue Dye Exclusion assay on human umbilical vein endothelial cells treated with up to 100 µg/mL of MWCNT (less than half the maximum concentration used in the present work) and have observed that a significant decrease in viability could be seen in association to concentration and time [83]. Another study used a Chinese hamster lung fibroblast cell line treated with 0-96 µg/cm^2 of SWCNT, and after counting the cells stained with Trypan Blue, the authors obtained a significant increase of cytotoxicity associated with exposure time and nanomaterial concentration [84].

Jacobsen *et al.*, though, used a cell counting assay for the assessment of viability of a mouse lung epithelial cell line exposed to 0-200 µg/mL of SWCNT and concluded that the cell viability did not display a significant decrease; furthermore, the reduced cytotoxicity these particles exhibited appeared to be reversible [85]. This may be an explanation for the results of the 24 hour exposure herein presented: the viability indeed decreased, but the cells replicated and the original number was restored. Nonetheless, the simple counting of cells is considered a not very reliable method for the assessment of cell viability. Besides, the number of inviable cells (assessed by trypan blue incorporation) found in each replicate of the assays was extremely small (usually 0, never more than 3; data not shown). The large standard deviations in several values are also an evidence of this unpredictability.

The Clonogenic assay was also performed on A549 cells, but not in BEAS-2B cells, as these could not form colonies. The results showed a quite consistent significant and dose-dependent decrease in viability in most doses of all the nanomaterials, which proves the evident cytotoxic effect these carbon nanotubes have in A549 cells. NM-401, in particular, caused an accentuated cytotoxicity. In the literature, it is possible to find a number of works that use this assay to analyze the cytotoxicity of carbon nanotubes. Herzog and colleagues, for example, have tested SWCNT on A549, BEAS-2B cells and human keratinocytes using this assay, as well as Casey *et al.* on A549 cells, and concluded that an increase of nanomaterial dose is accompanied by a decrease in number and size of the colonies [62, 86].

Other assays, such as the analysis of lactate dehydrogenase (LDH) or a colorimetric cell proliferation assay (MTT or MTS assay), can also be performed for the evaluation of the cytotoxicity of the nanomaterials. Kim *et al.* have obtained results consistent with the ones presented above: MWCNT were tested in a normal human bronchial epithelial cell line (concentration: 0.01-0.1%) with the cell proliferation kit WST-1, and the authors discovered that the particles were extremely toxic [87]. Two studies performed by Patlolla *et al.* using MWCNT (diameter: 15-30 nm; length: 15-20 µm) on normal human dermal fibroblast cells using MTT and LDH release assays showed a significant decrease in cell viability from 40 to 400 µg/mL [88, 89], which is consistent to the range of concentrations used in this project (0-256 µg/mL). Simon-Deckers and colleagues used several cytotoxicity assays in the same study, in order to analyze the influence of MWCNT (0-100 µg/mL) on A549 cells: MTT, XTT (3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) and LDH; these assays produced positive results, suggesting cytotoxicity of these particles; no association with physical characteristics or impurities could be made [82].

The micronucleus assay, besides from yielding genotoxicity data, also gives information concerning cytotoxicity, through the determination of the proliferation and the replication indexes. Statistical analysis was performed on these results; in this section, only one of these indexed will be explored (RI), due to the fact that the information provided by the two are similar. In this work, BEAS-2B cells' RI increased after

exposure to NM-400. In A549, on the other hand, the only significant change was caused by NM-401, and represented a decrease of RI.

One of the possible conclusions of the nanomaterial dispersion analysis was that the dispersion protocol used in this project may have influenced the results, since the particles formed masses quickly after sonication, and the cells were exposed to large agglomerates/aggregates of nanofibers as well as to the individualized particles. The possible outcome of this fact is that the cells were being physically damaged by very large and strong clusters, and not only by thin fibers that can possibly penetrate cellular membranes and influence the intracellular medium [90]. This theory may explain the results obtained in the Viability assays, both the (mostly) negative through cell counting and the positive in clonogenic assay. The large masses of fibers deposited over the cells during a long period of time may, in fact, damage the cells and prevent them from surviving and replicating. In the clonogenic assay, due to the reduced number of cells in the plate wells from the beginning, this cellular damage and destruction may compromise irreversibly the capacity of cells to divide and, consequently, the number of colonies formed, and their size. Using cell counting/trypan blue exclusion assay, on the other hand, this influence may not be so clear, since the number of plated cells is much larger, the exposure length is shorter and the dead cells may be washed away in the trypsinization of the culture, never reaching the counting step. This theory is in agreement with the results found in the literature, as other authors have documented the influence of cell density on the cytotoxicity of MWCNT, stating that the viability of the cells increases in higher cell densities [90].

In addition, it has been discovered that fibrous particles such as CNT can damage cell membranes, suggesting that the entrance in the cells is not always performed through endocytosis: there are reports of nanotubes causing holes in the membranes of the cells (possibly by membrane lipid peroxidation [91-94]), through which they have access to the cytoplasm. Indeed, some authors have not found membranes surrounding the agglomerates of nanomaterials inside the cells, discovering that they are free in the cytoplasm, which suggests the uptake of the particles was not performed via endocytosis [94, 95]. Even when the uptake of the fibers is done through endosomes of phagosomes, these structures are very fragile, as the particles can pierce the encompassing membranes and injure them, interfering with its function and integrity [90, 92, 93]. After entering the cells, the fibers may travel to any organelle or, alternatively, stay in the cytoplasm; this fact has not yet been confirmed [93]. DiGiorgio and collaborators have found morphological alterations in the nuclear envelope, which may indicate interaction of the fibers with the nucleus of the cells [91]. It has been stated also that the cells display a texturized membrane with ruffles and microvilli in the cell surface; after exposure to the carbon nanotubes, the surface morphology had changed and these properties were lost, suggesting the influence of the fibers in the cellular membranes [90, 93]. These morphology alterations in the membrane of the cells may play a very important part in the possible cytotoxicity of CNT, due to the fact that some lung cells (A549, for example) secrete lung surfactant, which may aid in the dispersion of the fibers or influence the contact of the particles with the

cells. By changing the number and morphology of the microvilli, the nanomaterials may affect the surfactant release as well, leading to the development of pathological conditions unrelated to the nanoparticle exposure [93]. This may be the explanation for the apparent higher susceptibility of A549 cells in relation to BEAS-2B. Cell surface morphology changes were also documented by other authors and in other cell lines exposed to different nanomaterials. Panessa-Warren and collaborators, as an example, tested a mix of carbon nanotubes (SWCNT, nanoropes and graphene, mostly) in human lung epithelial cells (NCI-H292) and human intestinal cells (Caco-2), and concluded that the percentage of apoptotic cells and cells in varying degrees of destruction increased, as well as injured microvilli and membrane damage [96]. Other authors, on the other hand, have reported that MWCNT do not influence the membrane integrity or permeability, such as Tabet *et al.*, who tested the influence of MWCNT in human alveolar epithelial cells (A549) and mesothelial cells (MeT5A) [97], and Di Giorgio and co-workers, who tested MW and SWCNT in mouse macrophages (RAW 264.7) [91]. There have also been reports of alterations of morphology on the inside of the cells, associated to an exposure to carbon nanotubes. Guo *et al.* demonstrated that these nanoparticles cause the formation of vacuoles in the cytoplasm of human umbilical vein endothelial cells, as well as the internalization of the fibers by these organelles; they have also noted a decrease in the average size of the cells, and an increase in the amount of apoptotic cells in comparison to the control [83].

However, as was stated in the **Material and Methods** chapter, there is also the possibility that these results (or at least, a part of them) are associated to impurities found in the nanomaterial solutions. Metallic contaminations are frequently found in this group of nanoparticles, and it has been suggested that the toxicity assigned to the CNT is actually caused by the trace amounts of iron, nickel, silver, aluminum, yttrium or others, coupled to the nanofibers [58]. Simon-Deckers and co-workers did not find this association, suggesting MWCNT caused cytotoxicity without the interference of iron impurities [82].

Another aspect that needs to be considered is the media used for the dispersion of the nanotubes and later, the exposure of the cells. As described in the **Material and Methods** chapter, the dispersion medium contains bovine serum albumin and the medium used to treat the cells with the nanomaterial solutions contains, in addition to a small amount of BSA that comes with the nanoparticles, fetal bovine serum. Some authors, as stated in the beginning of this chapter, have tested the influence of proteins in the nanomaterial toxicity, stating that the presence of serum can influence the uptake and the transport of the particles inside the cells. The proteins present in the serum may bind to the fibers and form a protein corona; this can decrease the toxicity of the nanotubes, as it influences the way they interact with the cells and other biological components [73, 74, 98].

In an attempt to correlate the viability results to the nanomaterials characteristics, regression analysis was performed on these associations. All the viability data was compared to the characteristics of the carbon nanotubes, but only the IC50 of A549 cells calculated in the clonogenic assay results section appeared to show associations. As was mentioned in the **Results** chapter, the IC50 of the NM-401 was not

calculated in the clonogenic assay, and therefore, this value will be determined by interpolation from the graph (Figure 24).

The cytotoxicity pattern of A549 cells seems to be directly associated to the average fiber length, diameter and surface area; these associations are represented in Figure 31. No association was found between the cytotoxicity results in this cell line and the dispersion quality of the CNT samples.

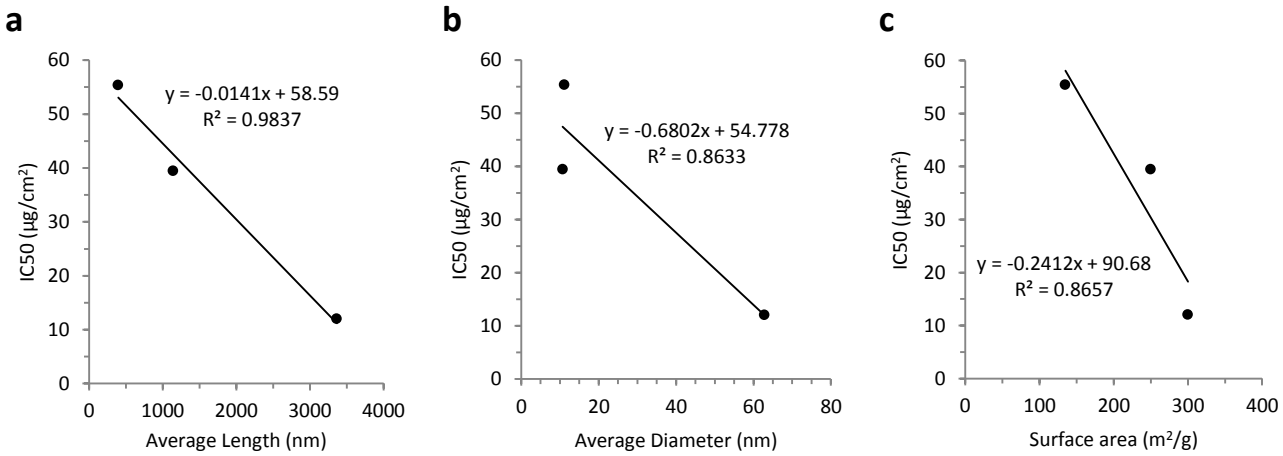


Figure 31. Association between the Clonogenic assay results and some of the tested MWCNT's characteristics. **a** – IC50 vs. average particle length; **b** – IC50 vs. average particle diameter; **c** – IC50 vs. surface area of the particles. The equations of the lines and respective correlation coefficient are also represented.

These results suggest that longer and thicker fibers are more cytotoxic, as well as those with a higher surface area. Longer MWCNT display greater cytotoxicity possibly due to their ability to cause a larger amount of damage to the membrane of the cells than shorter ones, injuring the microvilli responsible for the secretion of lung surfactant (as mentioned above). The diameter and surface area of the carbon nanotubes are related to their capacity to interact with biological tissues, as the interaction of the nanomaterials with proteins and other molecules present inside the organism is heavily influenced by the composition of the nanoparticle's surface; the resulting protein corona may, therefore, change over time, depending on the location of the particles inside the cells and inside the organism in general [99]. Indeed, there are reports that CNT can bind to serum proteins, which allows the particles to be more efficiently dispersed and internalized by the cells [99].

Simon-Deckers and colleagues, however, in their study concerning toxicity and accumulation of oxide and carbon nanoparticles in A549 cells, concluded that MWCNT's length does not influence the particle's toxicity; besides, the authors determined that only nanotubes with lengths of less than 3 µm actually enter the cells [82]. This suggests that NM-401 did not enter the cells; the positive cytotoxicity results may be caused, then, by membrane damage, microvilli destruction or physical aggression.

The lack of data concerning the influence of NM-400 on A549 cells creates the possibility that these associations may not be completely accurate. Further studies are necessary in order to clarify this issue.

4. Genotoxicity

An aspect that needs to be considered in the toxicity studies of nanomaterials is that the results from one assay or from the exposure of one given cell line cannot be extrapolated to other nanomaterials or cell systems, due to the fact that every particle has unique characteristics and properties and interact with biological molecules differently. Also, the particles fabricated in laboratories for research purposes may have different characteristics (such as surface properties, coating, charge, etc.) from those used in industrial applications. This may lead to erroneous conclusions concerning the toxicological profile of nanomaterials, as a large percentage of the toxicity results (either negative or positive) are possibly due to the external properties of the particles (as mentioned previously in this chapter) [97]. In addition, one of the biggest challenges in the comparison of results from the works documented in the literature is the discrepancies in terms of concentrations and metrics in the assays using nanomaterials. The metrics used in the studies are an essential aspect to be considered in the analysis of the results, as the concentration of the nanomaterial solutions, due to the fact that nanoparticles are solid and insoluble, should be measured in particle number, density or surface area instead of being based on mass. This is important because the characteristics of the particles are crucial in their toxicity; therefore this different way of expressing concentration is directly related to the particle's biological effect [100].

In this study, several endpoints were explored in order to test the genotoxicity of three carbon nanotubes in pulmonary tract cell lines. First, the comet assay was performed to analyze whether the nanoparticles caused primary damage in DNA – either single or double strand breaks or alkali-labile sites. This assay was performed in both cell lines, and with two exposure periods (3 and 24 hours). The second genotoxicity assay that was employed to analyze these nanoparticles was the micronucleus assay, which was used to confirm if the nanomaterials induced chromosome abnormalities, such as fragmentation or loss. It was performed in BEAS-2B and in A549 cells, and with the same nanomaterials as in the comet assay; all the exposure times were 48 hours. This assay has been validated as a strong indicator of genomic instability. Moreover, micronuclei, as well as the chromosomal abnormalities detected in this assay are good biomarkers of carcinogenic events [64].

The genotoxicity results are summarized in Table 8; note that, as before, the results of the analysis of NM-403 are also presented in this table, even though the actual assays were not performed during this study.

Table 8. Summary of the genotoxicity results.

Cell Line		BEAS-2B			A549		
Assay		Micronucleus	Comet		Micronucleus	Comet	
Exposure		48 hours	3 hours	24 hours	48 hours	3 hours	24 hours
Nanomaterials	NM-400	-	-	-	NP	NP	NP
	NM-401	-	-	-	+	-	-
	NM-402	-	-	-	+	-	-
	NM-403	-	NP	-	-	-	-

The results of the genotoxicity of the nanomaterials were mainly negative, with two exceptions in the micronucleus assay, in A549 cells.

In the conventional comet assay in BEAS-2B, the only significant results were those of NM-403 after an exposure of 24 hours. Although significantly different from the control, they consist in a very slight increase in the percentage of DNA in tail. For this reason, these results were not considered biologically relevant and, therefore, are not discussed further in this section. In A549 cells, the results are mainly in accordance to those of BEAS-2B. In this case, NM-401 caused an increase in DNA damage in the two highest concentrations after an exposure of 24 hours, but the results exhibit a large standard deviation; for this reason, these results do not show statistical significance in terms of difference from the negative control. The addition of FPG to this assay did not cause a relevant alteration in the results in either cell line. The results from the exposure of A549 and BEAS-2B cells to the nanomaterials led to similar results; however, identically to the conventional comet assay results, NM-401 displayed an apparent toxicity, represented by a line with an increasing slope. Due to the large deviation of values, these results are not significant either. It is possible to note that the percentage of DNA in the comet tail in both cell lines is slightly higher than those from the conventional comet, meaning that there was the production of a low level of oxidative damage. These results are in accordance with some of the studies found in the literature, but show discrepant results in comparison to others. The work from Cavallo *et al.*, as an example, used Comet assay (with and without FPG addition) on A549 cells, and determined that MWCNT caused an increase in direct DNA damage with an increase in CNT concentration [93], unlike what was described in this work's results. However, the MWCNT analyzed by those authors had lengths ranging from 0.5–200 µm, which is very superior to the range studied in this work. Furthermore, those authors report a lack of significant oxidative lesions, which is in agreement to the results described in this study; in some concentrations of the particles, though, the authors discovered evidence of some degree of oxidative stress, such as the development of blebs due to the interaction of the MWCNT with the microvilli at the surface of the cells [93].

DiGiorgio *et al.*, on the other hand, performed a comet assay on a mouse macrophage cell line (RAW 264.7) exposed to 1-50 µg/mL of MWCNT (with a diameter of 10–30 nm and a length of 0.5–50 µm) for 24

hours, and determined that there was production of DNA damage and formation of ROS, not dependent of CNT concentration [91]. Ghosh and collaborators performed a comet assay on human lymphocytes and on bone marrow cells from Swiss albino male mice (*Mus musculus*) exposed to MWCNT (diameter: 7-15 nm, length: 0.5-200 μm; concentrations: 0-10 μg/mL for the human lymphocytes and 2-10 mg/kg body weight for the mice); the authors discovered a significant production of DNA damage by the CNT on only one dose in the *in vitro* system, and in two doses in the *in vivo* exposure. No dose-response association could be made in either case. The authors proposed nanomaterial agglomeration as a possible explanation for this, as the actual concentration of particles to which the cells were exposed were not what was expected [101]. Ema and colleagues used Crl/CD(SD) rats to test MWCNT (diameter: 44 nm, length: 2.7 μm; concentrations: 0.04-1 mg/kg body weight) with comet assay, in several exposure conditions; it was possible to determine that none of the concentrations and none of the exposure conditions produced positive results, which led to the conclusion that MWCNT did not have capacity to cause genotoxicity *in vivo* [102]. Migliore and co-workers used a murine alveolar macrophage cell line (RAW 264.7) in a comet assay (with and without FPG and EndoIII), in order to test the genotoxicity of MWCNT (110-170 nm of diameter, 5-9 μm of length; concentrations: 0.01 to 100 μg/mL) in two exposure times (2 and 24 hours). Without the use of enzymes, after 2 hours of treatment the comet tails were not different from the control, and after 24 hours, were significantly different only in the highest doses; when the enzymes were employed, positive results arose in the lowest two concentrations in terms of oxidized purines and in the highest concentration in terms of oxidized pyrimidines. The authors suggest several explanations, such as the presence of impurities in the CNT or structural defects of the particles [103]. Also, Lindberg *et al* tested a mixture of SW and MWCNT (average diameter: 1.1 nm; length: 0.5–100 μm; concentrations: 1-100 μg/cm^2, or 3.8-380 μg/ml) in BEAS-2B cells, and noted an increase in DNA damage associated with the increase in concentration of particles; besides, the authors observed that longer exposure periods produced higher levels of damage than shorter ones [75].

As was mentioned previously, nanomaterials may have the capacity to induce the production of ROS, due to their characteristics, such as size and surface area. Indeed, nanoparticles can pierce the cellular membranes and interact with the organelles present in cytoplasm, possibly disrupting biochemical processes and reactions. The generation of ROS by nanomaterials may happen directly, due to their interaction with the cellular organelles and consequent production of free radicals or, alternatively, indirectly, due to the interference of the particles with oxidant and antioxidant processes [104]. Oxidative stress may lead to inflammation, as well as structural and functional modifications of biological components such as proteins or nucleic acids, which increases the possibility of genome defects and cell death [58, 105]. This may be due to the physical damage caused by the particles on the exterior membrane of the cells and/or to incomplete endocytosis and consequent release of the nanomaterials in the cytoplasm (possibly reaching, entering and damaging the nucleus) [91].

This study did not confirm the generation of oxidative stress in pulmonary tract cells by the exposure to the tested CNT. In the literature, however, it is possible to find conflicting reports related to this. Müller *et al.* determined that the exposure of A549 and immune system cells (human monocyte-derived macrophages and dendritic cells) to 30 µg/mL of SWCNT (20 nm diameter) did, in fact, induce the production of ROS and generation of oxidative stress, both in the individual cell cultures and in the triple cell co-culture [95]. Another example is a work already cited in this document, performed by Guo and co-workers who tested MWCNT in human umbilical vein endothelial cells for the production of ROS and concluded that these particles induced oxidative stress, which was likely to be related to the observed cytotoxicity and apoptosis [83]. Jacobsen and collaborators tested SWCNT in a mouse lung epithelial cell line, and discovered a significant generation of ROS, which decreased in the highest CNT concentrations; the authors suggested, as an explanation for this fact, the agglomeration of the particles, which logically increases with the concentration [85]. Karlsson and colleagues tested the effects of the exposure to MWCNT (diameter: 110-170 nm; length 5-9 µm; concentrations: 1- 40 µg/cm^2 or 2-80 µg/mL) in A549 cells, and observed that the generation of ROS in this case did not seem to increase [106].

Regarding the micronucleus assay, the genotoxicity results are different in the two cell lines: in BEAS-2B cells, only one of the materials (NM-403), in a single dose, caused a significant induction of micronuclei, while in A549, all three nanoparticles produced significantly positive data, and in several concentrations. This suggests that these two cell lines must possess critical differences in their characteristics, making one much more susceptible to MWCNT than the other. The differences observed between the results of the two assays (comet and micronucleus) may be due to a specific mode the nanomaterials may have of producing damage or to different sensitivities between the assays [75].

These discrepancies in the micronucleus assay results are a common feature found in the literature. Lindberg and colleagues, for example, tested a mixture of SW and MW carbon nanotubes (concentrations: 1-100 µg/cm^2, or 3.6-360 µg/mL) on BEAS-2B cells, and the results were mainly positive for induction of micronuclei; similarly to the results of the comet assay, the amount of damage increased with the concentration of nanomaterial. These authors also commented on a common problem associated to the use of these nanomaterials: the analysis of the results at higher doses was sometimes problematic due to the interference of masses of carbon nanotubes with the observation of the cells [75]. Kisin and colleagues studied SWCNT (average diameter: 1–10 nm) and carbon nanofibers (diameter: 50–200 nm, very variable length) in Chinese hamster lung fibroblast cells (V79) with comet and micronucleus assays. The results showed induction of genotoxic effects by both particles associated with the increasing nanomaterial concentration, but a lack of oxidative stress; besides, the particles displayed the same level of induction of micronucleus. Similarly to what was reported in other studies, the metallic impurities present in the nanomaterial samples were appointed as a possible reason for the results [107]. Kim *et al*. tested the biological response of ICR mice exposed to MWCNT (diameter: 10-15 nm; length 20 µm; intraperitoneal

administration) with an *in vivo* micronucleus assay. The main conclusions of that study were that, due to the deposition of the particles in the abdominal cavity of the mice without translocation to other organs or blood vessels, neither cytotoxicity nor micronucleus induction could be observed [108].

Even though the results from the A549 exposure to the nanomaterials produced mostly positive results in terms of micronucleus induction, it is not clear if these are consequences of aneugenic or clastogenic processes. Loss of chromosomes (aneuploidy) can be explained by interaction of the nanotubes with tubulin from the mitotic spindle, or with proteins involved in the segregation of the chromosomes in metaphase, disrupting the cell division. Breakage of chromosomes, on the other hand, can often be associated to the formation of DNA adducts and to oxidative stress. Metals are common causes of generation of ROS, due to their interaction with biological molecules and release of ions. For this reason, the presence of impurities in the solutions of nanomaterials is usually appointed as a probable reason for the toxicity of the particles; differences in metallic contaminants or coatings on the nanotubes are, therefore, factors that influence the outcome of most assays, and possible causes for the confusion surrounding the cyto- and genotoxicity of nanomaterials [107].

The two genotoxicity assays performed in this work have different endpoints, detecting different kinds of damage. The comet assay detects small DNA lesions that may be repairable – single or double strand breaks, easily repaired by specific enzymes; micronucleus assay, on the other hand, detects irreversible effects on DNA – chromosomal breaks or losses, as stated above. Aneugenic but not clastogenic compounds, for this reason, produce positive results in the micronucleus assay, but not in the comet assay; some discrepancies in this project may be explained by this theory [109]. Nanomaterials and, more specifically, multi-walled carbon nanotubes, may cause preferentially aneugenic effects, not producing positive results in the comet assay. Hartmann and co-workers tested several chemical compounds with comet and micronucleus assays, and noted that some of them caused significant micronucleus induction, but not an increase in the amount of DNA in the comet tails; two of these compounds were well-known aneugens, and the results coincides with the explanation above [109]. These authors also suggested another explanation for these inconsistencies between the results of the two assays: the impact of cytotoxicity on the results. This theory is based on the possibility that damaged or unviable cells that are further subjected to injury in the form of genotoxicity may be destroyed, and remain present in the comet assay slides, interfering with the reliability of the results [109]. For this reason, the cytotoxicity results described and discussed above are essential in the analysis of the genotoxic potential of MWCNT; although, this represents some controversy, due to the discrepant results obtained in the two cytotoxicity assays performed. According to cell counting/trypan blue exclusion assay, the nanomaterial concentrations used are perfectly adequate, not causing cytotoxicity on either of the cell lines; according to the clonogenic assay, on the other hand, the MWCNT caused severe damage on A549 cells and, for this reason, the

concentrations employed in the assays should not have been used. Explanations for these differences have been explored in the previous section.

The proposed reasons for the differences in the results, as well as the different endpoints explored in the assays suggest that the best course of action in genotoxicity testing is the use of more than one assay in the analysis of the potential of a particle to induce DNA damage; in cytotoxicity testing, as has been mentioned above, this principle also applies. For this reason, none of the results presented and discussed above should be regarded as inadequate or inaccurate.

As was explored in the cytotoxicity section, the results of the genotoxicity assays may be related to the physical and chemical characteristics of the nanomaterials, as well as to the dispersion homogeneity of the CNT's samples. In order to explore this option, the micronucleus assay results of the A549 cells exposure to the MWCNT were correlated to the known characteristics of the particles, and to their dispersion state. The only possible association was between the number of micronucleated binucleated cells and the aspect ratio of the particles (presented in Figure 32). The comet assay results were not subjected to this regression analysis, due to the fact that they were mostly negative in both cell lines and, therefore, whatever association that would arise would not be very significant.

As was mentioned previously, these regressions may not be entirely accurate due to the lack of genotoxicity information regarding NM-400, which was not tested in this cell line.

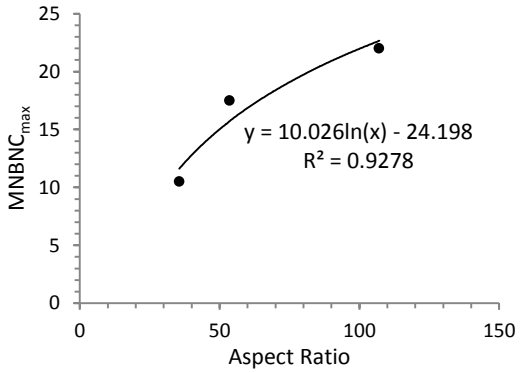


Figure 32. Association between the maximum number of micronucleated binucleated A549 cells (MNBNC$_{max}$) following exposure to the tested MWCNT and the aspect ratio of the particles. The equation of the line and correlation coefficient are also represented.

The aspect ratio is a ratio between the average length and diameter of the carbon nanotubes; in Figure 33, a representation of the four carbon nanotubes analyzed in this project can be found. In this project, it was noticed that a higher aspect ratio may be associated to a higher genotoxicity. A study mentioned previously in this work, by Kim and colleagues, presented different conclusions: the authors compared the influence of high and low aspect ratio CNT, and discovered that this property does not seem to interfere in the toxicity of the particles [108]. However, since that work was performed on an *in vivo*

system (ICR mice), with intraperitoneal administration, and the particles appeared to be contained in the abdominal cavity instead of penetrating the organs, the authors' conclusions concerning the association between the toxic potential of the fibers and their aspect ratio may not be extrapolated to the *in vitro* situation described in this study.

Figure 33. Visual representation of the four carbon nanotubes analyzed in this project. Scale: 1 cm=200 nm.

These results suggest that it may be possible to make the association between the physical characteristics and the toxicity of nanomaterials; however, to be able to clearly make this link, a larger amount of related nanomaterials should be tested in the future. In addition, it is very inaccurate to extrapolate the toxicity profile of one particle to any other, even if the characteristics of the materials are similar. This highlights the importance of studying every particle individually, instead of assuming similar biological behaviors and toxicities for a group of related nanomaterials [32].

Conclusions

This study has compared several carbon nanotubes in terms of toxicity profile, and attempted to relate this data to their physical characteristics. This association was not frequent or strong in any case, due to the lack of more nanomaterials available for comparison, but it may contribute to the present scientific knowledge concerning nanotoxicology.

The results of this work pointed to the absence of any detectable cyto- or genotoxicity of any of the nanomaterials tested in human bronchial epithelial cells. Concerning the other cell line tested, pulmonary epithelial cells, the results reveal a considerable cytotoxic potential of NM-401, NM-402 and NM-403, as well as a great ability of NM-401 and NM-402 to cause chromosome instability, as evidenced by the micronucleus assay. This study also added to the already existing notion that not all toxicity assays are adequate for the study of nanomaterials, as was evident by the comparison of the results of the two cytotoxicity assays performed.

In terms of future perspectives, there are some aspects that need further investigation, such as:

a) It is necessary to develop new technical methodologies specific for the testing of nanomaterials, as well as to validate the existing ones, in order to more accurately study the toxic potential of nanoparticles;

b) *In vitro* testing results should be supported by short term *in vivo* data as well, in order to analyze the influence of the organism and the immune system on the metabolism of the particles, instead of considering solely the target organ in the toxic effects of the nanomaterials;

c) Other endpoints should be explored, such as the production of ROS or the induction of mutations in the DNA, with the aim of better understanding the mechanism of toxicity of the particles;

d) All the published studies should be accompanied by the general characteristics of the nanomaterials, so that the obtained results can be compared with those of other studies;

e) Other projects with the same goal as this one – associating nanomaterials' characteristics to their toxic potential – must analyze a larger range of nanoparticles with comparable properties, in order to more accurately make this association.

In general, these results, complemented by those of the other partners of NANoREG project, will contribute greatly for the safety assessment of nanotechnology.

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Annexes

Annex A. Tables of the Results of the Tests for the Validation of the Methods – EMS

In the next two tables (A1 and A2), the results of the viability and genotoxicity assays performed on BEAS-2B and A549 cells exposed to EMS will be presented.

Table A1. Viability of BEAS-2B and A549 cells exposed to EMS.

Concentration (mM)	Viability (%) ± SD			
	BEAS-2B		A549	
	1h Exposure	3h Exposure	1h Exposure	3h Exposure
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.75	114.56 ± 8.61	NA	113.10 ± 14.11	NA
1	NA	155.90 ± 36.01	NA	129.57 ± 20.06
2	NA	82.54 ± 19.89	NA	64.42 ± 14.96
5	127.50 ± 14.92	76.48* ± 2.10	112.26 ± 26.18	72.12* ± 4.08
10	101.20 ± 13.68	105.40 ± 50.78	93.15 ± 22.95	71.27* ± 7.99
20	114.43 ± 18.02	NA	103.73 ± 27.37	NA
30	137.60* ± 5.24	NA	68.27* ± 9.52	NA
40	108.65 ± 4.55	NA	72.12* ± 4.08	NA

0 – negative control; SD – standard deviation; * - significantly different from control ($p \leq 0.05$, Student *t*-test); NA – Not applicable.

Table A2. Results of the comet assay on BEAS-2B and A549 cells exposed to EMS.

	Concentration (mM)	BEAS-2B		A549	
		1h	3h	1h	3h
DNA without FPG (Mean percentage of DNA in tail ± SD)	0	1.17 ± 0.79	1.17 ± 0.79	8.18 ± 3.28	8.18 ± 3.28
	0.75	2.36 ± 0.62	NA	20.42 ± 10.67	NA
	1	NA	4.15 ± 2.56	NA	18.11 ± 7.32
	2	NA	6.87 ± 3.74	NA	33.07 ± 5.68
	5	10.92 ± 6.56	20.81 ± 6.67	26.70 ± 4.22	49.11 ± 5.61
	10	15.75 ± 5.08	40.01 ± 12.19	50.04 ± 12.24	67.26 ± 5.01
	20	34.39 ± 14.27	NA	54.02 ± 7.74	NA
	30	51.19 ± 18.06	NA	66.33 ± 12.46	NA
	40	63.95 ± 9.43	NA	72.91 ± 8.63	NA
DNA with FPG (Mean percentage of DNA in tail ± SD)	0	4.39 ± 1.33	4.39 ± 1.33	12.31 ± 3.85	12.31 ± 3.85
	0.75	8.83 ± 1.02	NA	39.52 ± 4.44	NA
	1	NA	18.96 ± 3.66	NA	57.61 ± 8.49
	2	NA	30.89 ± 13.99	NA	64.04 ± 4.67
	5	35.58 ± 12.91	58.20 ± 4.66	63.11 ± 4.52	62.09 ± 8.32
	10	49.27 ± 19.94	75.49 ± 3.57	78.23 ± 1.69	70.24 ± 13.26
	20	71.57 ± 5.63	NA	64.95 ± 2.59	NA
	30	76.16 ± 8.92	NA	71.24 ± 9.10	NA
	40	76.38 ± 7.45	NA	78.46 ± 4.69	NA
Oxidative Lesions (Oxidative lesions ± SD)	0	3.22 ± 0.54	3.22 ± 0.54	4.13 ± 3.66	4.13 ± 3.66
	0.75	6.47 ± 1.37	NA	19.10 ± 7.75	NA
	1	NA	14.81 ± 6.20	NA	39.50 ± 13.66
	2	NA	24.02 ± 17.54	NA	30.97 ± 8.13
	5	24.67 ± 19.21	37.38 ± 10.82	36.41 ± 4.63	12.98 ± 11.62
	10	33.52 ± 24.16	35.49 ± 13.73	28.20 ± 10.72	2.98 ± 15.58
	20	37.17 ± 9.59	NA	10.93 ± 5.27	NA
	30	24.97 ± 10.67	NA	4.91 ± 4.73	NA
	40	12.43 ± 5.82	NA	5.55 ± 4.79	NA

0 – negative control; SD – standard deviation; * - significantly different from control (p≤0.05, One-Way ANOVA).

Annex B. Tables of the Results of the Viability Assays - MWCNT

In this section, tables B1-B4 contain the results of the viability assays performed on both cell lines, after exposure to the MWCNT. The results of the cell counting coupled to trypan blue exclusion assay, the clonogenic assay, and the two indexes calculated in the micronucleus assay are presented.

Table B1. Viability of BEAS-2B and A549 cells exposed to the studied MWCNT, analyzed using cell counting.

Concentration (µg/cm^2)	Viability ± SD								
	BEAS-2B							A549	
	NM-400		NM-401		NM-402		NM-403 §	NM-401	
	3h Exposure	24h Exposure	3h Exposure	24h Exposure	3h Exposure	24h Exposure	24h Exposure	3h Exposure	24h Exposure
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
16	129.74 ± 45.79	86.69 ± 11.42	110.96 ± 8.06	77.03* ± 5.93	106.39 ± 65.36	117.34 ± 37.27	NA	96.26 ± 28.38	95.62 ± 16.44
32	95.14 ± 15.45	77.27 ± 16.09	83.09 ± 28.19	70.00 ± 18.72	50.42 ± 23.81	96.39 ± 28.88	63.90 ± 41.70	89.97 ± 63.25	96.06 ± 7.62
64	107.16 ± 30.59	99.17 ± 11.80	98.31 ± 36.68	62.58* ± 1.42	58.52 ± 47.40	87.65 ± 32.91	97.90 ± 14.70	83.84 ± 34.39	68.00* ± 1.89
128	99.18 ± 11.87	79.84* ± 0.10	87.58 ± 47.56	61.95* ± 9.96	67.62 ± 17.01	76.67 ± 24.67	122.50 ± 41.20	106.29 ± 45.94	80.41 ± 42.06
EMS 0.75 mM	96.43 ± 41.47	83.65 ± 16.94	96.43 ± 41.47	83.65 ± 16.94	116.76 ± 61.25	80.36 ± 42.03	NA	127.72 ± 24.77	107.07 ± 4.35

0 – negative control; EMS – ethyl methanesulfonate (positive control); SD – standard deviation; * - significantly different from control (p≤0.05, Student *t*-test); NA – Not applicable; § - results from NANOGENOTOX project but included in the results of this work for comparison purposes.

Table B2. Viability of A549 cells exposed to the tested MWCNT, analyzed using the clonogenic assay.

Concentration (µg/cm^2)	A549								
	NM-401			NM-402 §			NM-403 §		
	Plating Efficiency ± SD	Surviving fraction ± SD	Cytotoxicity ± SD	Plating Efficiency ± SD	Surviving fraction ± SD	Cytotoxicity ± SD	Plating Efficiency ± SD	Surviving fraction ± SD	Cytotoxicity ± SD
0	61.60 ± 3.39	1.00 ± 0.00	0.00 ± 0.00	62.67 ± 6.60	1.00 ± 0.00	0.00 ± 0.00	58.00 ± 5.66	1.00 ± 0.00	0.00 ± 0.00
1.25	NA	NA	NA	61.00 ± 16.50	0.97 ± 0.26	2.66 ± 26.33	59.33 ± 0.94	1.02 ± 0.02	-2.30 ± 1.63
2.5	NA	NA	NA	63.33 ± 0.94	1.01 ± 0.02	-1.06 ± 1.5	50.67 ± 3.77	0.87 ± 0.07	12.64 ± 6.50
7.5	NA	NA	NA	61.33 ± 0.94	0.98 ± 0.02	2.13 ± 1.50	56.33 ± 4.24	0.97 ± 0.07	2.87 ± 7.31
16	22.40* ± 5.66	0.36* ± 0.07	63.83* ± 7.19	NA	NA	NA	NA	NA	NA
22.5	NA	NA	NA	61.67 ± 8.01	0.98 ± 0.13	1.60 ± 12.79	58.00 ± 11.31	1.00 ± 0.20	0.00 ± 19.51
30	NA	NA	NA	71.33 ± 2.83	1.14* ± 0.05	-13.83* ± 4.51	33.00 ± 5.19	0.57* ± 0.09	43.10* ± 8.94
32	15.00* ± 1.98	0.24* ± 0.02	75.70* ± 1.88	NA	NA	NA	NA	NA	NA
62.5	NA	NA	NA	23.33 ± 3.77	0.37* ± 0.06	62.77* ± 6.02	31.00 ± 6.13	0.53* ± 0.11	46.55* ± 10.57
64	14.40* ± 5.09	0.23* ± 0.07	76.82* ± 6.99	NA	NA	NA	NA	NA	NA
125	NA	NA	NA	3.00 ± 0.47	0.05* ± 0.01	95.21* ± 0.75	11.67 ± 3.30	0.20* ± 0.06	79.89* ± 5.69
128	13.80* ± 1.98	0.22* ± 0.02	77.65* ± 1.98	NA	NA	NA	NA	NA	NA
MMC 0.1 µg/mL	3.60* ± 0.57	0.06* ± 0.01	94.17* ± 0.60	8.33 ± 0.47	0.13 ± 0.01	86.70 ± 0.75	6.67 ± 4.71	0.11 ± 0.08	88.51 ± 8.13

0 – negative control; MMC – mitomycin C (positive control); SD – standard deviation; * - significantly different from control (p≤0.05, Student *t*-test); NA – Not applicable; § - results from NANOGENOTOX project but included in the results of this work for comparison purposes.

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Table B3. Results from the indexes calculated in the micronucleus assay on BEAS-2B cells exposed to the studied MWCNT.

Concentration (µg/cm^2)	BEAS-2B							
	NM-400		NM-401		NM-402		NM-403 §	
	CBPI ± SD	RI ± SD	CBPI ± SD	RI ± SD	CBPI ± SD	RI ± SD	CBPI ± SD	RI ± SD
0	1.96 ± 0.04	100.00 ± 0.00	1.96 ± 0.04	100.00 ± 0.00	2.01 ± 0.03	100.00 ± 0.00	2.23 ± 0.07	100.00 ± 0.00
16	2.11 ± 0.00	115.73 ± 4.79	1.98 ± 0.02	102.30 ± 2.32	2.13 ± 0.06	113.94 ± 8.61	NA	NA
32	2.16 ± 0.01	120.95 ± 6.36	1.98 ± 0.04	102.46 ± 0.93	2.12 ± 0.09	125.47 ± 10.68	2.33 ± 0.06	104.19 ± 4.47
64	2.23 ± 0.03	128.42 ± 2.34	1.90 ± 0.05	93.68 ± 0.51	2.17* ± 0.06	127.80 ± 8.30	2.31 ± 0.11	102.70 ± 8.31
128	2.30 ± 0.06	135.68 ± 12.07	1.95 ± 0.03	98.85 ± 8.08	2.19* ± 0.07	104.48 ± 8.00	2.35 ± 0.10	105.52 ± 8.16
MMC 0.15 µg/mL	1.37 ± 0.02	38.56 ± 0.75	1.37 ± 0.02	38.56 ± 0.75	1.25* ± 0.13	27.12* ± 11.58	1.26* ± 0.07	20.40* ± 5.33

0 – negative control; MMC – mitomycin C (positive control); SD – standard deviation; * - significantly different from control (p≤0.05, Fisher's exact test); NA – Not applicable; § - results from NANOGENOTOX project but included in the results of this work for comparison purposes.

Table B4. Results from the indexes calculated in the micronucleus assay on A549 cells exposed to the studied MWCNT.

Concentration (µg/cm^2)	A549					
	NM-401		NM-402		NM-403 §	
	CBPI ± SD	RI ± SD	CBPI ± SD	RI ± SD	CBPI ± SD	RI ± SD
0	1.71 ± 0.02	100.00 ± 0.00	2.03 ± 0.09	100.00 ± 0.00	1.95 ± 0.06	100.00 ± 0.00
1.25	NA	NA	1.90 ± 0.09	87.00 ± 8.55	1.93 ± 0.11	98.10 ± 11.25
2.5	NA	NA	2.02 ± 0.01	99.09 ± 0.78	1.91 ± 0.00	95.54 ± 0.21
7.5	NA	NA	1.95 ± 0.05	92.20 ± 4.94	1.95 ± 0.00	100.36 ± 0.13
16	1.69 ± 0.00	97.23 ± 4.02	NA	NA	NA	NA
22.5	NA	NA	2.01 ± 0.04	97.93 ± 4.04	2.02 ± 0.06	107.66 ± 6.06
30	NA	NA	1.99 ± 0.12	96.18 ± 11.5	1.94 ± 0.06	99.34 ± 5.96
32	1.67 ± 0.01	94.61 ± 1.42	NA	NA	NA	NA
62.5	NA	NA	2.00 ± 0.04	96.81 ± 3.81	1.96 ± 0.01	101.39 ± 1.56
64	1.65 ± 0.00	92.55 ± 3.66	NA	NA	NA	NA
125	NA	NA	2.12 ± 0.01	108.30 ± 0.50	1.96 ± 0.02	101.33 ± 1.76
128	1.59 ± 0.01	83.97 ± 1.46	NA	NA	NA	NA
MMC 0.15 µg/mL	1.39 ± 0.03	55.18 ± 5.50	1.99 ± 0.01	38.16 ± 0.32	1.46 ± 0.16	39.37 ± 7.04

0 – negative control; MMC – mitomycin C (positive control); SD – standard deviation; * - significantly different from control (p≤0.05, Fisher's exact test); NA – Not applicable; § - results from NANOGENOTOX project but included in the results of this work for comparison purposes.

Annex C. Tables of the Results of the Genotoxicity Assays – MWCNT

This section contains the results of the genotoxicity assays: comet and micronucleus, performed on BEAS-2B and A549 cells exposed to MWCNT (tables C1 to C4).

Table C1. Genotoxicity results of comet assay on BEAS-2B cells exposed to the studied MWCNT.

	Concentration ($\mu g/cm^2$)	BEAS-2B						
		NM-400		NM-401		NM-402		NM-403 §
		3h Exposure	24h Exposure	3h Exposure	24h Exposure	3h Exposure	24h Exposure	24h Exposure
Without FPG (Mean percentage of DNA in tail ± SD)	0	3.27 ± 1.16	3.32 ± 2.52	3.27 ± 1.16	3.32 ± 2.52	1.82 ± 1.03	2.24 ± 0.53	1.77 ± 1.73
	16	2.20 ± 1.09	3.01 ± 0.66	1.39 ± 0.55	2.47 ± 0.97	2.21 ± 1.01	3.29 ± 0.57	NA
	32	2.90 ± 2.22	2.81 ± 0.12	2.50 ± 0.47	2.34 ± 0.48	2.26 ± 0.93	2.84 ± 1.59	3.58* ± 1.88
	64	1.85 ± 0.34	2.65 ± 1.11	2.19 ± 0.27	2.01 ± 0.83	2.11 ± 0.31	2.31 ± 0.43	2.45 ± 0.73
	128	1.86 ± 0.68	2.10 ± 0.66	1.93 ± 1.13	2.67 ± 0.98	2.25 ± 1.09	2.14 ± 0.52	2.92* ± 1.44
	EMS 0.75 mM	6.42* ± 1.83	22.02* ± 9.30	6.42* ± 1.83	22.02* ± 9.30	3.17 ± 1.02	11.30* ± 2.51	8.47* ± 1.73
With FPG (Mean percentage of DNA in tail ± SD)	0	3.52 ± 0.77	5.17 ± 0.66	3.52 ± 0.77	5.17 ± 0.66	4.05 ± 1.01	4.44 ± 1.98	4.08 ± 0.84
	16	3.55 ± 1.34	4.88 ± 1.55	3.52 ± 0.74	4.40 ± 1.40	3.09 ± 2.29	6.63 ± 2.83	NA
	32	3.10 ± 0.74	5.35 ± 1.92	5.21 ± 3.90	3.85 ± 0.74	3.85 ± 2.77	4.21 ± 1.36	5.45 ± 1.09
	64	3.60 ± 1.30	3.70 ± 1.05	3.73 ± 0.45	3.75 ± 1.04	4.17 ± 1.19	4.60 ± 1.16	4.61 ± 2.44
	128	4.00 ± 0.83	6.99 ± 2.54	4.24 ± 1.95	3.85 ± 0.96	3.39 ± 2.02	3.18 ± 0.25	5.04 ± 1.15
	EMS 0.75 mM	13.39 ± 8.83	36.06* ± 14.47	13.39 ± 8.83	36.06* ± 14.47	14.96* ± 5.14	23.99* ± 3.90	NA
	H_2O_2 10 mM	NA	NA	NA	NA	NA	NA	71.39* ± 9.98
Oxidative Damage (Oxidative Lesions ± SD)	0	0.25 ± 0.71	1.85 ± 2.42	0.25 ± 0.71	1.85 ± 2.42	2.23 ± 1.7	2.19 ± 1.86	1.54 ± 1.63
	16	1.35 ± 1.26	1.86 ± 2.03	2.13 ± 1.18	1.93 ± 1.63	0.88 ± 2.16	3.34 ± 2.31	NA
	32	0.21 ± 2.74	2.54 ± 1.87	2.7 ± 4.03	1.51 ± 0.96	1.59 ± 3.51	1.37 ± 2.77	2.35 ± 0.87
	64	1.75 ± 1.61	1.05 ± 1.22	1.54 ± 0.67	1.74 ± 0.84	2.07 ± 1.47	2.29 ± 0.98	0.50 ± 2.02
	128	2.13 ± 1.37	4.89 ± 2.06	2.31 ± 2.5	1.18 ± 1.27	4.71 ± 7.51	3.77 ± 5.48	1.97 ± 1.23
	EMS 0.75 mM	6.97 ± 8.68	14.04* ± 6.10	6.97 ± 8.68	14.04* ± 6.1	10.58* ± 4.38	12.93* ± 7.72	NA
	H_2O_2 10 mM	NA	NA	NA	NA	NA	NA	14.68 ± 8.26

0 – negative control; EMS – ethyl methanesulfonate (positive control); H_2O_2 – hydrogen peroxide (positive control); SD – standard deviation; * - significantly different from control ($p \leq 0.05$, One-Way ANOVA); NA – Not applicable; § - results from NANOGENOTOX project but included in the results of this work for comparison purposes.

Table C2. Genotoxicity results of comet assay on A549 cells exposed to the studied MWCNT.

	Concentration ($\mu g/cm^2$)	A549					
		NM-401		NM-402 §		NM-403 §	
		3h Exposure	24h Exposure	3h Exposure	24h Exposure	3h Exposure	24h Exposure
Without FPG (Mean percentage of DNA in tail ± SD)	0	2.90 ± 0.54	3.91 ± 0.29	2.48 ± 0.22	1.06 ± 0.08	2.48 ± 0.22	1.06 ± 0.08
	2.5	NA	NA	2.65 ± 0.49	1.51 ± 0.44	1.25 ± 0.09	1.87 ± 0.92
	8	NA	NA	1.74 ± 0.63	1.80 ± 0.31	2.86 ± 0.23	2.47 ± 0.15
	16	4.50 ± 1.58	3.68 ± 0.97	2.05 ± 0.04	1.72 ± 0.32	3.75 ± 1.41	3.12 ± 1.17
	32	5.99 ± 1.86	3.91 ± 0.62	2.64 ± 0.02	2.98 ± 1.76	4.25 ± 0.53	2.29 ± 0.15
	64	6.03 ± 2.10	8.63 ± 5.91	3.97 ± 2.85	2.14 ± 0.21	3.52 ± 0.87	5.03 ± 0.45
	128	4.42 ± 0.99	16.26 ± 16.32	2.08 ± 0.94	2.17 ± 0.90	5.01 ± 0.53	4.59 ± 0.79
	EMS 0.75 mM	6.82* ± 1.70	14.92* ± 3.64	NA	NA	NA	NA
With FPG (Mean percentage of DNA in tail ± SD)	0	7.64 ± 1.41	6.57 ± 3.21	2.76 ± 1.42	3.49 ± 1.14	2.76 ± 1.42	3.49 ± 1.14
	2.5	NA	NA	2.79 ± 1.59	4.13 ± 0.21	4.91 ± 1.47	3.67 ± 0.68
	8	NA	NA	3.12 ± 0.79	2.87 ± 1.02	4.05 ± 2.01	2.72 ± 0.44
	16	8.48 ± 1.98	7.39 ± 1.72	2.89 ± 0.73	3.60 ± 0.60	3.65 ± 0.27	3.02 ± 0.20
	32	7.02 ± 2.86	8.18 ± 1.30	4.11 ± 0.79	NA	4.93 ± 1.59	4.17 ± 1.96
	64	7.91 ± 0.87	11.01 ± 6.78	5.27 ± 1.28	4.05 ± 0.22	6.60 ± 1.46	6.06 ± 2.73
	128	5.87 ± 0.64	15.70 ± 10.63	3.55 ± 0.65	4.40 ± 0.31	6.28 ± 0.19	5.06 ± 2.70
	EMS 0.75 mM	28.70* ± 7.21	47.28* ± 15.10	NA	NA	NA	NA
	H_2O_2 10 mM	NA	NA	74.38 ± 1.29	71.65 ± 0.44	74.38 ± 1.29	71.65 ± 0.44
Oxidative Damage (Oxidative Lesions ± SD)	0	4.74 ± 1.59	2.65 ± 3.02	0.27 ± 1.64	2.43 ± 1.22	0.27 ± 1.64	2.43 ± 1.22
	2.5	NA	NA	0.14 ± 2.08	2.62 ± 0.65	3.65 ± 1.38	1.79 ± 1.59
	8	NA	NA	1.39 ± 1.42	1.07 ± 0.71	1.19 ± 2.24	0.26 ± 0.28
	16	3.98 ± 0.83	3.71 ± 1.93	0.84 ± 0.77	1.88 ± 0.92	-0.10 ± 1.14	-0.10 ± 1.37
	32	1.03* ± 1.80	4.27 ± 1.83	1.48 ± 0.81	NA	0.68 ± 2.12	1.88 ± 2.11
	64	2.17 ± 1.77	2.37 ± 4.39	1.29 ± 4.13	1.90 ± 0.02	3.08 ± 2.32	1.03 ± 3.19
	128	1.45* ± 1.33	-0.55 ± 7.85	1.47 ± 1.59	2.23 ± 0.60	1.27 ± 0.71	0.47 ± 1.91
	EMS 0.75 mM	21.88* ± 8.71	32.36* ± 12.51	NA	NA	NA	NA
	H_2O_2 10 mM	NA	NA	12.17 ± 7.16	64.26 ± 7.61	12.17 ± 7.16	64.26 ± 7.61

0 – negative control; EMS – ethyl methanesulfonate (positive control); H_2O_2 – hydrogen peroxide (positive control); SD – standard deviation; * - significantly different from control ($p \leq 0.05$, One-Way ANOVA); NA – Not applicable; § - results from NANOGENOTOX project but included in the results of this work for comparison purposes.

Table C3. Results from the micronucleus assay on BEAS-2B cells exposed to the studied MWCNT (MNBNC/1000 BNC ± SD).

Concentration (µg/cm^2)	BEAS-2B			
	NM-400	NM-401	NM-402	NM-403 §
0	4.00 ± 2.83	4.00 ± 2.83	8.25 ± 1.77	12.25 ± 2.22
16	3.00 ± 0.00	4.00 ± 1.41	7.00 ± 1.41	NA
32	5.50 ± 3.54	3.00 ± 1.41	5.00 ± 2.12	6.75* ± 3.50
64	3.00 ± 0.00	1.00 ± 0.00	6.25 ± 2.47	10.75 ± 6.99
128	4.00 ± 2.83	7.00 ± 1.41	6.75 ± 0.35	8.00 ± 4.97
MMC 0.15 µg/mL	31.00* ± 0.00	31.00* ± 0.00	48.25* ± 10.25	85.25* ± 15.00

0 – negative control; MMC – mitomycin C (positive control); SD – standard deviation; * - significantly different from control (p≤0.05, Fisher's exact test); NA – Not applicable; § - results from NANOGENOTOX project but included in the results of this work for comparison purposes.

Table C4. Results from the micronucleus assay on A549 cells exposed to the studied MWCNT.

Concentration (µg/cm^2)	A549		
	NM-401	NM-402	NM-403
0	6.50 ± 0.71	10.50 ± 9.19	12.50 ± 3.54
1.25	NA	5.00 ± 2.83	9.50 ± 2.12
2.5	NA	7.50 ± 0.71	10.00 ± 1.41
7.5	NA	8.50 ± 2.12	6.00* ± 2.83
16	3.50 ± 2.12	NA	NA
22.5	NA	15.50 ± 7.78	5.50* ± 4.95
30	NA	12.00 ± 4.24	7.00 ± 1.41
32	5.00 ± 4.24	NA	NA
62.5	NA	22.00* ± 1.41	10.50 ± 4.95
64	6.00 ± 0.00	NA	NA
125	NA	20. 00* ± 1.41	10.00 ± 1.41
128	17.50* ± 2.12	NA	NA
MMC 0.15 µg/mL	50.00* ± 1.41	95.50* ± 6.36	105.00* ± 15.56

0 – negative control; MMC – mitomycin C (positive control); SD – standard deviation; * - significantly different from control (p≤0.05, Fisher's exact test); NA – Not applicable; § - results from NANOGENOTOX project but included in the results of this work for comparison purposes.

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